Attorney's Docket No. 5051-445 PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: Moyer et al. Examiner: S. Bausch
Serial No.: 09/912,072 Group Art Unit: 1634
Filed: July 24, 2001
For: IDENTIFICATION OF POINSETTIA CULTIVARS

Supplemental Declaration of Dr. James W. Moyer under 37 C.F.R. § 1.132

I, James W. Moyer, do hereby declare and state as follows:

1. My credentials were presented in a previous declaration submitted May 23, 2005.

2. I am a named inventor on U.S. Patent Application No. 09/912,072 (*hereinafter* "the '072 application").

3. At the time the work in the '072 application was carried out, AFLP technology had not been used to measure genetic diversity in poinsettia or in any species closely related to poinsettia. The application of AFLP technology to any particular plant species is considered uncertain and in light of this, to assess the relevance of the Loh et al., Barcaccia et al., Sukhwinder et al. and Barker et al. publications to poinsettia, it is further relevant to consider how distantly related poinsettia is to rice, willow, *Pelargonium* and *Caladium*. The taxonomic relationship of poinsettia as compared with these plants is shown in Appendix A.

4. All of the plants at issue are in the Division *Magnoliophyta,* the division to which all flowering plants belong. Two of the plants referenced, rice and caladium, are further classified as monocots (Class *Liliopsida*), while three, pointsettia, willow and geranium, are dicots (Class *Magnoliopsida*).

5. Rice is further classified into the Subclass *Commelinidae,* Family *Poaceae,* and Genus *Oryza*. The *Commelinidae* contains an enormous diversity of plants including rushes and sedges, cereals, pineapples, ginger, and arrowroot. Therefore, it is nearly impossible to generalize findings with regard to one member of the *Commelinidae* such as a grass (e.g., maize or rice) to another member such as a bromeliad (e.g., a pineapple).

6. Caladiums are in the Subclass *Aracidae.* The *Aracidae* includes the palms, duckweed and caladium. *Caladium* is further classified into the Family *Araceae* (aroid), and Genus *Caladium.* The aroid group includes edible plants as well as those that are poisonous and includes philodendron, calla lily, Mexican breadfruit and taro. Thus, there is an enormous diversity of plants in the Subclass *Aracidae* and it would be difficult to generalize regarding one member such as duckweed with another member such as palm or philodendron.

7. Within the class *Magnoliopsida*, or the dicots, willow (*Salix*) is further classified into the Subclass *Dilleniidae.* The *Dilleniidae* is once again a very diverse grouping including, for example, the mustard family (e.g., broccoli), the heath family (e.g., rhododendron and blueberry) and the cucurbit family (e.g., cucumber, squash).

Willow is further classified into the Family *Salicaceae* and the genus *Salix*. Once again, it would be very difficult to generalize findings about a member of this diverse Subclass, such as willow or broccoli, to another member such as blueberry or squash.

8. Both *Pelargonium* and poinsettia are in the Subclass *Rosidae*. This is an incredibly diverse group of plants with 108 families and 58,000 species. Some examples of plant families that fall within the *Rosidae* include the carrot family, the apple family, the legume family (e.g., pea), and the dogwood family. In addition, the *Rosidae* subclass also includes the geranium family to which *Pelargonium* belongs and the very large and diverse euphorbia or spurge family (approximately 300 genera and 7,500 species) to which poinsettia belongs. (Park et al., Int J. Plant Sci. 161:425-434 (2000); C.L. Porter, *Taxonomy of Flowering Plants*, W.H. Freeman & Co., 472 pp., p.338, (1967)). *Pelargonium* is further classified into the Genus and Species *Pelargonium peltatum*. Poinsettia is in the Genus *Euphorbia,* which has been described as one of the largest and most complex genera of flowering plants with about 1600 species. *Id.*. Clearly, it is nearly impossible to generalize findings about one member in the Subclass *Rosidae,* such as carrot to another member such as dogwood. This would be difficult even within the single Genus *Euphorbia,* of which poinsettia is a member, due to its great size and diversity.

9. Plant scientists interested in determining genetic relationships between and within plant cultivars, varieties or species would be well-aware of the distant relationship between poinsettia and the plants studied in the cited publications, and would not find our achievement in poinsettia obvious in light of results in a tree such as willow, a cereal plant such as rice, an aroid plant such as caladium, or even geranium. It is simply not the case that AFLP analysis in any other plant, particularly distantly related plants such as those in the cited publications, would suggest application of AFLP analysis in poinsettia. A scientist in this field would at most think to try such a technique but would enter into the research without any expectation of success. This would be particularly true in the case of poinsettia since it is known to have a very narrow genetic base (*See,* prior Moyer Declaration submitted May 23, 2005).

10. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

James W. Moyer, Ph.D. 1/25/06
Date

Attachment: Appendix A

APPENDIX A. Taxonomic relationships between poinsettia and the plant species in the cited references.

Kingdom: *Planta*
- Subkingdom: *Tracheobionta* – vascular plants
  - Superdivision: *Spermatophyta* – seed plants
    - Division: *Magnoliophyta* – flowering plants
      - Class: *Magnoliopsida* – dicotyledons
        - Subclass: *Dilleniidae*
          - Order: *Salicales*
            - Family: *Salicaceae*
              - Genus: *Salix* – Willow
        - Subclass: *Rosidae*
          - Order: *Geraniales*
            - Family: *Geraniaceae*
              - Genus: *Pelargonium* – Geranium
          - Order: *Euphorbiales*
            - Family: *Euphobiaceae*
              - Genus: *Euphorbia* – Poinsettia
      - Class: *Liliopsida* – monocotyledons
        - Subclass: *Arecidae*
          - Order: *Arales*
            - Family: *Araceae*
              - Genus: *Caladium*
        - Subclass: *Commelinidae*
          - Order: *Cyperales*
            - Family: *Poaceae*
              - Genus: *Oryza* – Rice

Int. J. Plant Sci. 161(3):425–434. 2000.

1058-5893/2000/16103-0010$03.00

A PHYLOGENETIC STUDY OF TRIBE EUPHORBIEAE (EUPHORBIACEAE)

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A phylogenetic investigation of a monophyletic lineage of spurge plants, tribe Euphorbieae, was conducted to elucidate evolutionary relationships, to clarify biogeographic patterns, and to reexamine the previous classification of Euphorbieae. Cladistic analyses of the 52 morphological characters of 61 species resulted in 2922 equally most parsimonious trees of 193 steps with a consistency index of 0.34. The strict consensus tree indicates genus *Anthostema* of subtribe Anthosteminae as a likely sister group to all other members of tribe Euphorbieae. The morphological data support a monophyletic origin of subtribe Euphorbiinae, but the subtribes Anthosteminae and Neoquillauminiinae did not form monophyletic groups. Although the previous taxonomic treatments within tribe Euphorbieae have supported the generic status of *Pedilanthus*, *Monadenium*, *Synadenium*, *Chamaesyce*, and *Elaeophorbia*, the results of this analysis do not support generic placement of them based on cladistic principles. Recognition of these groups as genera results in *Euphorbia* becoming a paraphyletic group. One solution to this problem in Euphorbieae is to divide the largest genus *Euphorbia* into several monophyletic genera and to keep the generic ranks for previously recognized genera. The distribution of basal endemic genera in Euphorbieae showed African and east Gondwanan affinities and strongly indicated that the ancestor of Euphorbieae originated prior to the breakup of Gondwanaland from an old group in Euphorbiaceae. However, some recent African taxa of *Euphorbia* should be interpreted by transoceanic dispersal from the New World ancestors.

Keywords: phylogeny, Euphorbieae, morphology.

Introduction

The reconstruction of phylogenetic relationships of organisms facilitates the testing of systematic hypotheses as well as organizing of species into a formal classification (Nelson and Platnick 1981; Funk and Brooks 1990; de Queiroz and Gauthier 1994). The phylogenetic relationships of tribe Euphorbieae have yet to be thoroughly examined using cladistic methods (Gilbert 1994; Webster 1994*b*). The diversity of the taxonomic treatments within this tribe is cumulatively a result of the tribe's large size, the profusion of intergrading and overlapping characters (Sherff 1940), and a tribal classification that occurred without a comprehensive study of the entire group and throughout the entire range of its species. Thus, testing of generic and infrageneric boundaries within tribe Euphorbieae based on cladistic principles is one of the fundamental tasks of concern to systematists who specialize in *Euphorbia* and Euphorbiaceae.

Tribe Euphorbieae (ca. 2000 species), one of the most distinctive and largest tribes in the Euphorbiaceae (Croizat 1938), is characterized by the possession of a unique cyathium that consists of a central carpellate flower and four or five groups of basal male-flower clusters (Croizat 1937; Dressler 1957; Webster 1994*b*). The tribe is widely distributed throughout the world, but the majority of species occurs in tropical and subtropical Africa and America. The most recently proposed classification of tribe Euphorbieae (Webster 1975, 1994*b*) recognizes three subtribes: Anthosteminae, Neoguillauminiinae, and a true cyathium–bearing Euphorbiinae. This classification has been based mainly on staminate calyx, petaloid appendage, and gland characters. Pax and Hoffman (1931) recognized 10 genera and represented their relationships with a phyletic graph (reconstructed in fig. 1; Pax 1924). In this phyletic graph, Pax recognized four groups and proposed that *Anthostema* and *Dichostemma* are placed in a close relationship; *Calycopeplus* was treated as the most closely related genus to a group of *Euphorbia* + *Elaeophorbia*, and *Synadenium*, *Stenadenium*, and *Monadenium* share a common ancestor. Pax may have overemphasized the shape of involucral glands, and the genera with continuous glands (i.e., *Synadenium*, *Monadenium*, and *Stenadenium*) were treated as an independent lineage—one different from the true cyathium–bearing *Euphorbia* in his diagram.

An alternative phyletic scheme, one that includes *Neoguillauminia* and *Pedilanthus*, was proposed by Croizat (1937), and it has contributed significantly to our understanding of the phylogenetic relationships and cyathium evolution of tribe Euphorbieae (fig. 1). Croizat (1937) noted the presence of a male or female calyx in *Anthostema*, *Dichostemma*, *Neoguillauminia*, and *Calycopeplus* and segregated them from the true cyathium genera that have glands attached to the rim of the involucre. He considered *Calycopeplus* to be closely related to *Euphorbia*. Additionally, he emphasized the zygomorphic cyathium of *Cubanthus* and *Pedilanthus* and treated them as groups outside of *Euphorbia*.

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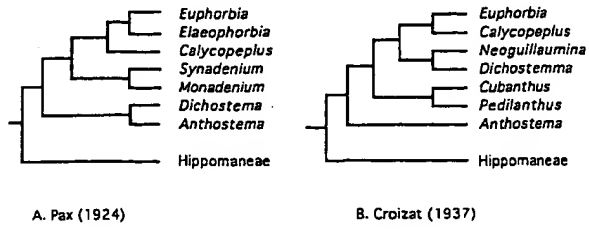

Fig. 1 Relationships within Euphorbieae proposed previously by Pax (1924) and Croizat (1937). Hypotheses of relationships are portrayed cladistically.

Within *Euphorbia* s.l., the New World endemics *Chamaesyce* and *Pedilanthus* have been treated as distinct genera or as closely related subgenera of subg. *Agaloma* based on their shared petaloid appendages, which appear around the involucral glands (Wheeler 1941; Dressler 1961). The species of *Chamaesyce* differ from the rest of the Euphorbiinae in that they have several unique characters, and they have been recognized as a genus by some authors (Croizat 1943; Webster 1994*b*). The polyphyly of *Euphorbia* s.l. was questioned based on the diverse origin of glands in Euphorbieae (Croizat 1938).

Cytological evidence (Hans 1973) indicates that within *Euphorbia* s.l., modern euphorbia species evolved from the primitive dendroid subg. *Esula* (x=6, 7, 8, 9, 10) (Perry 1943) or subg. *Euphorbia* (x=10) through an aneuploid or polyploid series (Webster 1967; Mehra and Choda 1978). *Anthostema* is distinguished from the rest of Euphorbieae by a distinct base number x=11. Common basic chromosome number (n=14) indicates a close relationship among native New World subg. *Agaloma*, *Poinsettia,* and *Chemaesyce* (Urbatsch et al. 1975).

A recent cladistic analysis of 33 morphological characters at the sectional level was conducted for New World subtribe Euphorbiinae (Park 1996). However, this phylogenetic analysis of New World Euphorbiinae did not support the generic recognition of *Chamaesyce* because it was nested within subg. *Agaloma* and because it required generic segregation for many groups within *Agaloma* (Park 1996). This analysis also did not strongly support the monophyletic origin of New World endemic petaloid-appendaged taxa and nested a monophyletic *Chamaesyce*, *Poinsettia,* and *Pedilanthus* within a paraphyletic *Agaloma*.

Using morphological characters, we conducted a phylogenetic investigation of spurge plants, tribe Euphorbieae, to elucidate phylogenetic relationships among the major taxonomic groups of Euphorbieae and to clarify biogeographic patterns among them. Additional objectives were to test the monophyly of subgenera proposed by Wheeler (1943) and by Carter and Radcliffe-Smith (1988) in *Euphorbia* s.l. and to examine whether New World petaloid-appendaged taxa form a monophyletic group.

Material and Methods

Monophyly, Terminal Taxa, and Outgroup Selection

The Euphorbieae have been accepted as a monophyletic group that is characterized by its unique cyathium (Croizat 1937; Webster 1967; Mehra and Choda 1978; Gilbert 1994). Recent phylogenetic analysis of subfamily Euphorbioideae strongly supported a monophyletic tribe Euphorbieae (K.-R. Park and A. Backlund, unpublished data) and its sister group relationships to species in tribe Hippomaninae + Hureae. Based on these analyses, two species of Hippomaninae, *Maprounea brasiliensis* and *Sebastiana brasiliensis,* were selected as outgroups for the ingroup taxa.

Although it is ideal to start a cladistic analysis with monophyletic species groups as terminal taxa in the analysis of large ingroups (Sanderson 1991), this was not possible in the case of Euphorbieae because no prior cladistic hypotheses of relationships within Euphorbieae were present, except for New World taxa. Thus, species were selected as terminal taxa, and at least one species of most higher taxonomic groups in Euphorbieae was chosen (Boissier 1862; Pax and Hoffman 1931; Carter and Radcliff-Smith 1988; Webster 1994*b*) in this analysis (appendix). Fifty-nine ingroup species were selected as terminal taxa.

Characters and Their States for Cladistic Analysis

Fifty-two qualitative characters are used in this analysis (table 1). Characters were obtained by direct examination of herbarium specimens and from the taxonomic literature.

Habit. In the Euphorbieae, most species outside of *Euphorbia* possess a woody habit with many stems (character 1). Within *Euphorbia* s.l., however, various forms of habit are present, even within the same subgenus. Trees or shrubs are quite common in subg. *Euphorbia*, whereas the woody condition is usually restricted to species occurring in insular or coastal habits in subg. *Chamaesyce* and *Esula* (Park 1996). Succulent stems (character 2) are very common in African endemic members of subg. *Euphorbia*, *Lacanthis*, *Eremophyton*, and genus *Elaeophorbia*; succulence is generally associated with deciduous leaves (character 12). With ternate stems and opposite phyllotaxy, stem articulation (character 3) occurs only in *Agaloma* sect. *Alectoroctonum* (Park 1996).

Inflorescence branching. The floral rays (character 7) of herbaceous species of Euphorbiniiae usually branch dichasially several times, and they are subtended by an equal number of leafy bracts. Some subtropical members of Euphorbiniiae possess a pseudoumbel dichasium at the tips of main stems. An umbel-dichasial inflorescence is present in most *Euphorbia* species; a thyrse (character 8) occurs only in some species in subg. *Agaloma* sect. *Alectoroctonum* and in *Dichostemma*. The presence of scattered rays (character 9) is considered to be ancestral within Euphorbieae, based on the previous analysis (Park 1996), but condensed or simple rays are restricted to *Euphorbia* subg. *Poinsettia*, a few species of subg. *Agaloma*, subg. *Tirucalli* and *Trichadenia*.

Leaves, bracts, and spines. The leaves (character 11) of Euphorbieae (in general) are alternate, whereas those of species of sect. *Zygophyllidium* in *Agaloma* and *Poinsettia* are opposite. Species of *Agaloma* sect. *Alectoroctonum* (*Euphorbia cotinifolia* and *Euphorbia leucocephala*) have verticillate leaves. Leaf margins of most species of subg. *Eremophyton*, *Poinsettia,* and *Chamaesyce* have serrate or dentate leaves (character 13), whereas remaining species in Euphorbieae have entire leaves as an ancestral condition. The equilateral bases

Table 1

Characters and Character States Used in Cladistic Analysis of Tribe Euphorbieae (Euphorbiaceae)

Character	State
1. Habit	0 = tree or shrub; 1 = perennial herb; 2 = annual herb
2. Stem type	0 = woody; 1 = semisucculent; 2 = succulent
3. Stem node	0 = nonarticulate; 1 = articulate
4. Branchlets	0 = absent; 1 = present
5. Fasciculate leaves	0 = absent; 1 = present
6. Branch shape in cross section	0 = cylindrical; 1 = angled
7. Umbel dichasium	0 = absent; 1 = present
8. Thyrse	0 = absent; 1 = present
9. Inflorescence ray	0 = scattered; 1 = condensed; 2 = simple
10. Monopodial terminal branch	0 = absent; 1 = present
11. Phyllotaxy	0 = alternate; 1 = opposite
12. Leaf persistence	0 = persistent; 1 = deciduous
13. Leaf margin	0 = entire; 1 = dentate or serrate
14. Leaf base symmetry	0 = equilateral; 1 = nonequilateral
15. Interpetiolar leafy stipule	0 = absent; 1 = present
16. Glandular stipule	0 = absent; 1 = present
17. Whorled bracts	0 = absent; 1 = present
18. Bract base shape	0 = inclined (elliptic); 1 = even (half-circle shape or deltoid)
19. Bract color	0 = green only; 1 = green and anthocyanic
20. Spine shield	0 = absent; 1 = present
21. Spine	0 = absent; 1 = present
22. Inflorescence position	0 = terminal; 1 = axillary
23. Cyathium	0 = absent; 1 = present
24. Male inflorescence	0 = enclosed by bract; 1 = not enclosed by bract
25. Male flower	0 = unorganized; 1 = group of four; 2 = group of five
26. Staminate calyx	0 = present; 1 = absent
27. Carpellate flower	0 = basal; 1 = apical
28. Carpellate calyx	0 = present; 1 = absent
29. Capsule surface	0 = smooth; 1 = verrucose
30. Capsule trichomes	0 = absent; 1 = present
31. Capsule shape	0 = three-lobed or rounded; 1 = triangular
32. Carpellate flower pedicel type	0 = straight; 1 = reflexed
33. Style tip	0 = undivided; 1 = divided
34. Style	0 = connate all of the length; 1 = free; 2 = connated at base
35. Appendage gland	0 = absent; 1 = present
36. Appendage shape	0 = round; 1 = deltoid; 2 = divided at margin; 3 = pectinate with acute tips
37. Gland shape	0 = rounded; 1 = crescent
38. Gland number	0 = four; 1 = five; 2 = one or two; 3 = more than five
39. Gland connation	0 = free; 1 = connate with ring shape
40. Gland surface	0 = flattened or folded; 2 = funnel- or cup-shaped
41. Gland position	0 = between staminate flower clusters; 1 = between involucral lobes
42. Involucral tube	0 = absent; 1 = 4-lobed; 2 = unlobed
43. Involucral lobe	0 = not petaloid; 1 = petaloid
44. Seed shape	0 = globose; 1 = angular
45. Seed dorsal view	0 = rounded; 1 = conical; 2 = rectangular
46. Seed caruncle	0 = present with round shape; 1 = present with cap shape; 2 = absent
47. Seed tubercula	0 = absent; 1 = present
48. Seed honeycomb	0 = absent; 1 = present
49. Seed horizontal ridges	0 = absent; 1 = present
50. Seed endocarp	0 = thick; 1 = thin
51. Pollen polar view	0 = round; 1 = deeply three-lobed
52. Stamen number	0 = more than one; 1 = one

of leaves are plesiomorphic in Euphorbieae, whereas the nonequilateral leaves (character 14) with interpetiolar stipules (character 15) are restricted to species of *Euphorbia* subg. *Chamaesyce* (Croizat 1943; Webster 1994*b*). Only *Monadenium*, some species of subg. *Esula*, and *Tirucalli* have semicircular or deltoid bracts, but in all other taxa, elliptic bases of bracts are predominant (character 18). Species having succulent stems with spiny outgrowths (character 21) are restricted to the African endemic subg. *Euphorbia*, subg. *Lacanthis*, and *Elaeophorbia*. The spine shield (character 20) surrounding the

base of leaves is an advanced character of subg. *Euphorbia* in *Euphorbia* s.l. (Carter 1994).

Staminate and carpellate flowers. The staminate calyx (character 26) of *Anthostema*, *Dichostemma*, and outgroup taxa of Euphorbieae is present, but it is lacking in most of the taxa within Euphorbieae. Subsequently, Webster (1994*b*) recognized subtribe Anthosteminae, including the above two genera, based mainly on this character. In some species of *Cubanthus* and *Euphorbia* subg. *Agaloma*, the vestigial forms of the staminate calyx apparently are present (Croizat 1937; Park 1998), but in this analysis, they are coded as lacking the staminate calyx. A pistillate calyx (character 28) is found in subtribes Anthosteminae, Neoguillauminiinae, and only *Cubanthus* of Euphorbiinae. All other taxa have no pistillate sepals, but they are well developed in outgroup taxa.

Involucral characters. Most of the members of the New World subtribe Euphorbiinae are characterized by petaloid appendages on the involucral glands (character 35). Wheeler (1939), Dressler (1957, 1961), and Webster (1967) hypothesized close relationships among *Euphorbia* subg. *Chamaesyce*, subg. *Agaloma*, and *Pedilanthus* based on this character. Two different gland positions (character 41) are known in Euphorbieae. The glands of subtribe Euphorbiinae are on the margin of the cyathia, which is opposite to the clusters of male flowers, whereas the glands of the two remaining subtribes are inserted between the clusters of male flowers. The glands of outgroups, subtribe Hippomaninae, occur on each side of the floral bracts enclosing the male flowers. Therefore, the alternative position of the glands in Anthosteminae and Neoguillauminiinae is a symplesiomorphic condition within the Euphorbieae.

Cladistic Analysis

Multistate characters were treated as unordered (Fitch 1971). The data sets were analyzed using maximum-parsimony algorithms available on the PAUP* version 4.0b2 (Swofford 1999) for the Macintosh. The matrix was analyzed using random taxon addition sequences followed by the efficient tree bisection-reconnection branch-swapping algorithm with MULPARS. A strict consensus tree was generated. In order to evaluate the stability of different branches in the obtained trees, a bootstrap analysis with 1000 replicates of data (using fast-heuristic search, MacClade version 3.0; Maddison and Maddison 1992) was used to explore character evolution and biogeographic pattern and to depict alternate tree topologies. An area cladogram was produced by replacing the name of taxa on the data matrix with their geographic occurrences (Platnick and Nelson 1978).

Results

Cladistic analyses resulted in 2922 equally most parsimonious trees of 193 steps with a consistency index of 0.34 and a retention index of 0.74. The strict consensus tree is represented in figure 2.

The strict consensus tree indicates genus *Anthostema* of subtribe Anthosteminae as a likely sister taxon to all other members of tribe Euphorbieae. The monophyly of subtribe Euphorbiinae, a true cyathium–bearing group, was supported by three synapomorphic characters, but subtribes Anthosteminae and Neoguillauminiinae did not form a monophyletic group (fig. 2). All trees supported sister-group relationships between subtribe Euphorbiinae and *Calycopeplus oligandrus* of Neoguillauminiinae and between *Cubanthus linearifolius* and the remaining taxa of Euphorbiinae. Within subtribe Euphorbiinae, the segregate genera *Pedilanthus*, *Monadenium*, *Synadenium*, and *Elaeophorbia* were nested within a paraphyletic *Euphobia* s.l.

The *Monadenium* + *Synadenium* clade forms a sister-group relationship with the Australian endemic *Euphorbia* subg. *Eremophyton* clade, including *Euphorbia parvicaruncula*, *E. stevenii*, *E. tannensis*, and *E. boöphthona* (fig. 2). Subgenus *Eremophyton* is paraphyletic.

The strict consensus tree indicated a sister-group relationship between *Elaeophorbia* and *Euphorbia* subg. *Lacanthis*, which in turn are sister to *Euphorbia* subg. *Euphorbia*. The New World endemic *Pedilanthus* is sister to the clade consisting of *Elaeophorbia*, *Euphorbia* subg. *Lacanthis*, and subg. *Euphorbia*.

This analysis supports the monophyly of five subgenera within *Euphorbia* s.l.: *Chamaesyce*, *Poinsettia*, *Tirucalli*, *Euphorbia*, and *Lyciopsis* (fig. 2). Three subg. *Agaloma*, *Eremophyton*, and *Esula* are found to be polyphyletic or paraphyletic. Only one additional step was required for subg. *Esula* to become monophyletic.

Discussion

Phylogeny and classification. A recent classification system (Webster 1994*b*) in tribe Euphorbieae recognized three subtribes and initially segregated Anthosteminae from Neoguillauminiinae + Euphorbiinae based on the presence of a staminate calyx in the Anthosteminae subtribe. Croizat (1937) suggested that *Anthostema* had basal placement within the tribe and implied that it was not closely related to *Dichostemma*. This analysis supported Croizat's hypothesis and indicated that the staminate calyx is the plesiomorphic condition within the tribe. In our analyses, monophyly of the Anthosteminae (Pax 1924; Webster 1994*b*), *Anthostema* and *Dichostemma*, required only one additional step, and this subtribe cannot be fully rejected based on this analysis.

Croizat (1937) also hypothesized a close relationship between *Euphorbia* and *Calycopeplus* and proposed *Calycopeplus* as an ancestor of *Euphorbia* s.l. This analysis supported Croizat's (1937) hypothesis, if *Cubanthus* is considered a member of *Euphorbia* s.l. Croizat treated *Cubanthus* and *Pedilanthus* as an independent lineage within the Euphorbieae, but our data and analysis did not support this relationship. In this study, *Pedilanthus* was nested within *Euphorbia* s.l. and formed a sister group to the African endemic clade consisting of *Elaeophorbia*, *Euphorbia* subg. *Lacanthis*, and *Euphorbia*. This result represented a departure from previous hypotheses related to the origin of *Pedilanthus*. Dressler (1957) and Park (1996) hypothesized that *Pedilanthus* was derived from the New World endemic subg. *Agaloma* sect. *Tricherostigma*. However, early interpretations of relationships (Boissier 1862; Millspaugh 1913; Croizat 1942) indicated a close affinity between *Cubanthus* and *Pedilanthus*.

Among genera of Euphorbieae, Pax (1924) proposed a close

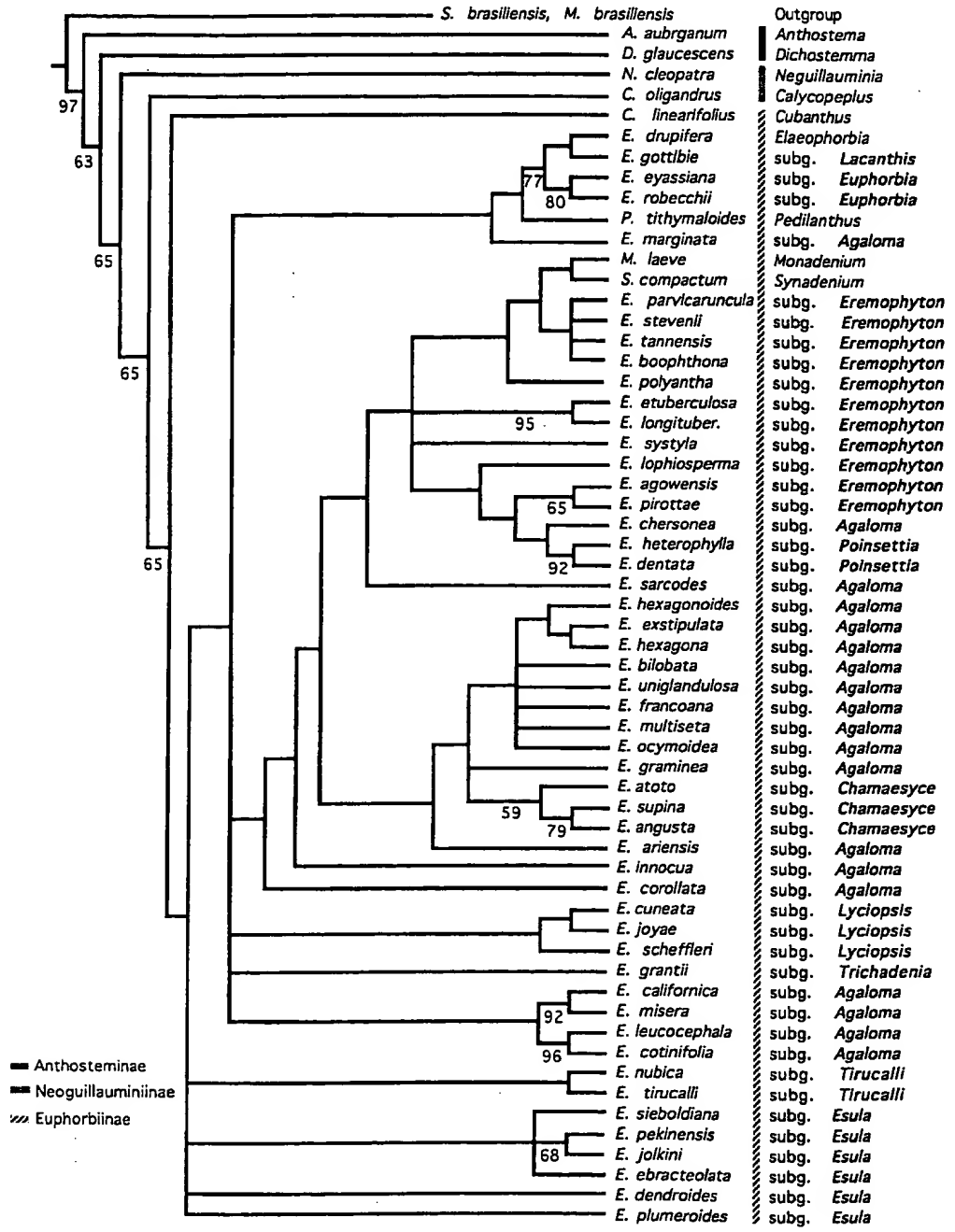

Fig. 2 Strict consensus tree of 2922 equally most parsimonious cladograms of tribe Euphorbieae. Genera and subgeneric names of *Euphorbia* are represented. Numbers below branches denote bootstrap values above 50%.

relationship between *Synadenium* and *Monadenium* and segregated them accordingly from *Calycopeplus*, *Euphorbia*, and *Elaeophorbia*. Our cladistic analysis supports this relationship and indicates common ancestry between these genera, but our analysis did not support segregation of the genera from *Euphorbia*, because they showed a close affinity with the *Euphorbia* subg. *Eremophyton* group.

Several workers recognized *Elaeophorbia* Stapf as a genus separate from *Euphorbia*, based on its unique indehiscent drupaceous fruit (Brown 1915; Pax and Hoffmann 1931; Hutchinson 1969; Carter and Radcliffe-Smith 1988). Recently, Evans and Kinghorn (1977), using diterpene profiles, rejected the recognition of *Elaeophorbia*. Our analysis of morphological characters placed *Elaeophorbia* as a sister group to *Euphorbia*

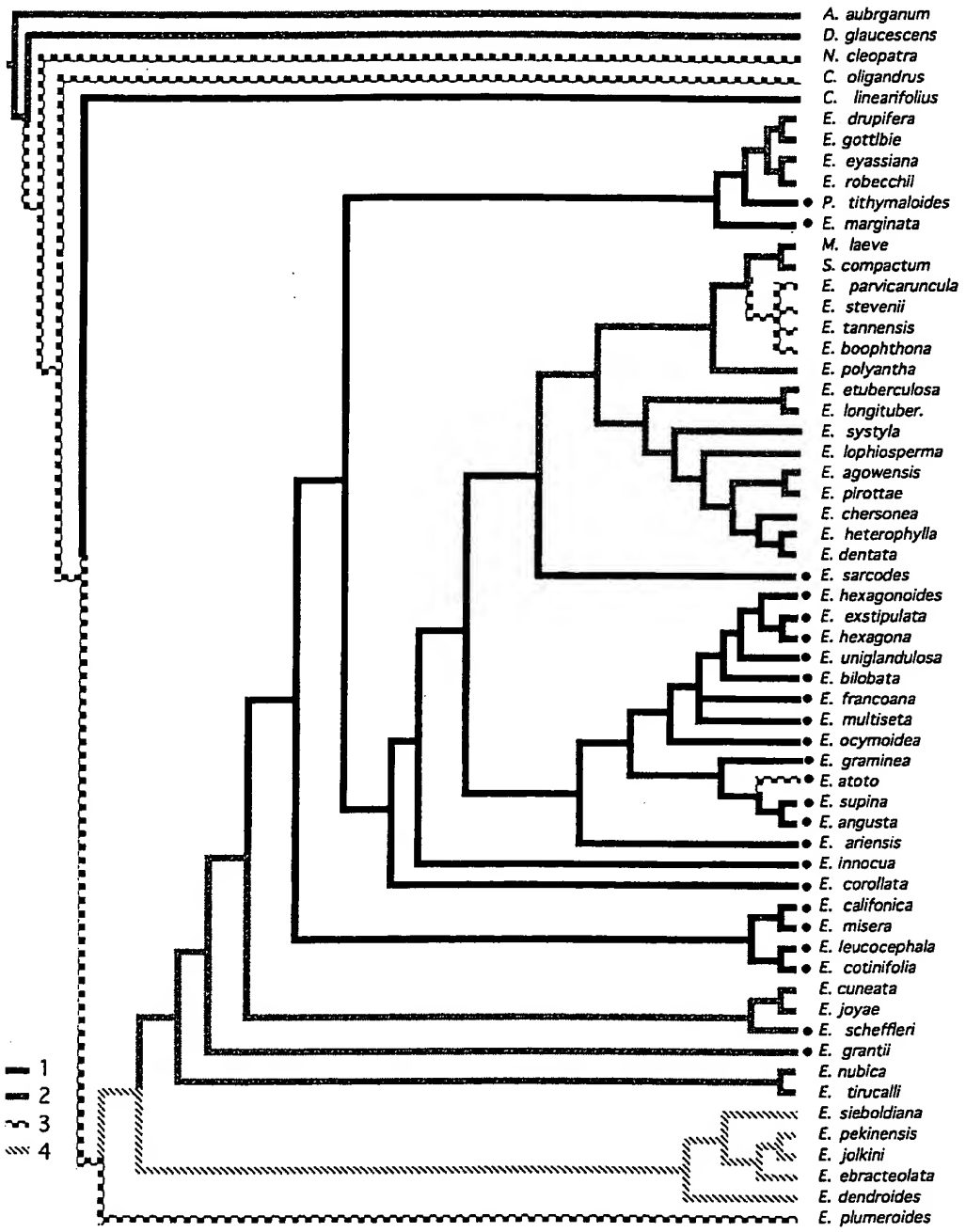

Fig. 3 One of the most parsimonious cladograms from morphological analysis and the geographic distribution of each species (*1* = America; *2* = Africa; *3* = Australasia; *4* = Asia). Closed circles represent species that have appendaged glands on the rims of the involucre.

subg. *Lacanthis*, one that was nested within the subg. *Euphorbia* + *Lacanthis* clade.

Within the genus *Euphorbia*, subg. *Euphorbia* is one of the most well recognized groups with a unique spine shield and pair of spines (Carter 1994). Gilbert (1987) and Carter (1994) hypothesized that subg. *Euphorbia* may have originated in Asia from a subg. *Tirucalli*–like ancestor. Carter (1994) also suggested that the calloused leaf scars of subg. *Tirucalli* should be homologous with the spine shield in subg. *Euphorbia*. The results of this study do not support the relationships proposed by Gilbert (1987) and Carter (1994), but they do strongly indicate the close relationships among subg. *Euphorbia*, *Lacanthis*, and *Elaeophorbia*.

Euphorbia subg. *Eremophyton* (Boiss.) Wheeler is a grouping of ca. 20 species native to Africa and Australia, and it is usually divided into two or three sections based on seed char-

acters (Boissier 1862; Hassall 1977; Carter and Radcliffe-Smith 1988). Carter and Radcliffe-Smith (1988) recognized only two sections: sect. *Pseudacalypa*, with a conical, ecarunculate seed, and sect. *Eremophyton*, with a cap-like carunculate seed. The presence of several independent lineages within subg. *Eremophyton* had been proposed by several authors (Boissier 1862; Croizat 1972; Radcliffe-Smith 1974). This study did not support the monophyly of subg. *Eremophyton*. However, the Australian endemic species of subg. *Eremophyton* formed a monophyletic group, which is in accord with the hypothesis proposed by Hassall (1977).

A close relationship among New World petaloid-appendaged groups of Euphorbiinae has been proposed by several monographers (Wheeler 1939; Dressler 1957, 1961; Webster 1967), but close relatives of the New World taxa were not identified. Our analysis indicated that the taxa with petaloid appendages were paraphyletic and shared a common ancestry with African endemic subg. *Trichadenia* and subg. *Lyciopsis* (fig. 3). If this relationship is supported, then the finger-like processes on the glands in the latter two subgenera are homologous to the petaloid appendages on the glands in New World taxa.

The close relationships of *Euphorbia* subg. *Chamaesyce* and the probable derivation from New World taxa have been indicated by the distribution of several characters: abortion of the main stem, a C_4 photosynthetic pathway, interpetiolar stipules, and nonequilateral leaves (Koutnik 1987). Wheeler (1941), Hurusawa (1954), and Webster (1967) hypothesized several models for the origin of *Chamaesyce* from *Euphorbia* subg. *Agaloma*, whereas Croizat (1943) insisted on an independent origin of *Chamaesyce* from *Euphorbia*. A recent cladistic analysis of New World Euphorbiinae (Park 1996) supported Norton (1900) and Webster's (1967) views that *Chamaesyce* is closely related to subg. *Agaloma* species such as *Euphorbia ipechacuanhae* and *Euphorbia innocua*. The results of our analysis strongly supported the origin of *Chamaesyce* within subg. *Agaloma*.

Dressler (1961) postulated that *Euphorbia* subg. *Poinsettia* was closely allied to subg. *Agaloma* sect. *Dichillum* Boiss. Park (1996) hypothesized that the condensed inflorescence of sect. *Petaloma* might be homologous with that of subg. *Poinsettia*, whereas Croizat (1942) speculated on a close relationships with African and Australian subg. *Eremophyton* species such as *Euphorbia crotonoides* and *Euphorbia eremophila*. This analysis, which includes Old World Euphorbieae, did not support a close relationship between subgenera *Poinsettia* and *Agaloma* but indicated subg. *Poinsettia* nested within the *Euphorbia* subg. *Eremophyton* clade, close to *Euphorbia agowensis* and *Euphorbia pirottae*.

Within a polyphyletic *Euphorbia* subg. *Agaloma*, this study also supported the monophyly and sister-group status of sections *Alectroctonum* (*Euphorbia cotinifolia* and *Euphorbia leucocephala*) and *Tricherostigma* (*Euphorbia californica* and *Euphorbia misera*).

Although previous taxonomic treatments within tribe Euphorbieae have recognized the generic status of *Pedilanthus*, *Monadenium*, *Synadenium*, *Chamaesyce*, and *Elaeophorbia*, the results of this analysis do not support generic recognition of these taxa. Recognition of these groups as genera results in *Euphorbia* becoming a paraphyletic group.

Biogeography. The Euphorbieae, the largest tribe in Euphorbiaceae, is broadly divided into two geographical areas: a Paleotropical distribution in primitive taxa, and a Neotropical and Eurasian distribution in derived members. Most authors strongly advocate a recent origin for Euphorbieae and accept long-distance dispersal to explain the worldwide patterns within the tribe (Webster 1994*a*). In this study, the distribution of basal endemic genera in Euphorbieae showed African and east Gondwanan affinities (fig. 3) and strongly indicated that the ancestor of Euphorbieae originated prior to the breakup of Gondwanaland from an old group in Euphorbiaceae.

The pattern within *Euphorbia* s.l. indicates that the origin and basal diversification occurred in Australasia and Asia and was followed by dispersal to Africa and the New World. *Euphorbia* subg. *Tirucalli* consists of ca. 30 species and shows disjunct distributions through the Paleotropical region in Madagascar, the Cape region, East Africa, and Indochina. The cladistic trees revealed that the species of the subg. *Tirucalli*, one of the primitive members of *Euphorbia* s.l., originated from the common ancestor with subg. *Esula* in Southeastern Asia and then dispersed to India and moved on to diversification in Africa (ca. 20 species) from Tertiary. The most recent African members of *Elaeophorbia* and *Euphorbia* (subg. *Eremophyton*, subg. *Euphorbia*, subg. *Lacanthis*) are independently introduced from the New World. Although the pattern observed in basal groups in Euphorbieae may be explained by Gandwanan vicariance, some recent *Euphorbia* s.l. should be interpreted based on transoceanic dispersal models (Webster 1994*a*).

Acknowledgments

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Appendix

Table A1

Data Matrix of Character States for 52 Morphological Characters Used in Cladistic Analysis of Euphorbieae

	1111111111222222222233333333334444444444555 12345678901234567890123456789012345678901234567890123456789012
Tribe Euphorbieae:	
Subtribe Anthosteminae:	
Genus *Anthostema* (2):	
A. aubrganum	00000000100000000000011010100000100?0100010000000000
Genus *Dichostemma* (3):	
D. glaucescens	00000001000000000000001010100100100?000001000?000001
Subtribe Neoguillauminiinae:	
Genus *Neoguillauminia* (1):	
N. cleopatra	00000000000000000000001011100000100?0300021000000001
Genus *Calycopeplus* (5):	
C. oligandrus	00000100101100000000011011100000100?0000121000000101
Subtribe Euphorbiinae:	
Genus *Cubanthus* (3):	
C. linearifolius	00000010100000000000001121100001120?0202120?2000101
Genus *Elaeophorbia* (1):	
E. drupifera	02000100000100000000111121110000100?0100120002000001
Genus *Monadenium* (50):	
M. laeve	11000000000000010100011121110001100?0210120121100101
Genus *Synadenium* (10):	
S. compactum	01000000000000010000001121110001100?021012000?100101
Genus *Pedilanthus* (15):	
P. tithymaloides	01000000100100010000011121110001121000001200002000101
Genus *Euphorbia* (1000):	
E. dendroides	00000010000000001100001121110001100?1000120000000101
E. plumerioides	00000010000000001000001121110001100?0100120000000101
E. sieboldiana	11000010000000001100001121110001100?1000120000000101
E. pekinensis	11000010000000001000001121111001100?0000120000000101
E. jolkini	11000010000000001000001121111001100?0000120000000101
E. ebracteolata	11000010000000001100001121110001100?0000120000000101
E. eyassiana	02010100100100010001111121110000100?0100120002100?1
E. robecchii	02010100100100010001111121110000100?0100120002000?1
E. gottlbie	02011100000100010010111121110000?100?0100120002000?1
E. nubica	00010010200100011100001121110001100?0000120000000?1
E. tirucalli	00010010200100011100001121110001100?0100120000000101
E. cuneata	00011010?00000110000011211101001000?0101120002000?1
E. joyae	00000010?00000110000011211101001000?0100120002000?1
E. scheffleri	00000010200000011000001121110000101301001200020001?1
E. grantii	00000010000000011100001121110001101300001200020001?1
E. etuberculosa	12000010000000011000001121110001100?0201120112001101
E. longituberculosa	12000010?00000110000011211100011000?0201120112001101
E. systyla	21000010?00000110000011211100011000?0000120112101101
E. polyantha	11000010000000011100001121110001100?0000120121100101
E. lophiosperma	11000010000010011000001121110101100?0000120112101101
E. agowensis	21000010010010011000001121110101110?0000120121100101
E. pirottae	2100001001000?0?000001121110101110?0000120121100101
E. parvicaruncula	21000000010110010000001121110001100?0000120121100101
E. stevenii	21000000010110010000001121110001100?0000120002100101
E. tannensis	21000000010110010000001121110001100?0000120121100101
E. boöphthona	21000000010110010000001121110001100?0000120121100101
E. innocua	110000?000000001?000001121110101101000001201020101 01
E. corollata	11000010000000011000001121110001101001001200020101 01
E. hexagonoides	21000000?10000100000011211100011111010012011210012?1
E. bilobata	21000000?10000100000011211101011112010012011201?1
E. exstipulata	21000000?10100100000011211101011110000012011010?1
E. hexagona	21000000?10100100000011211100011111010012000010?101
E. californica	00011000200000000000001121110001101001001200020101?1

Table A1

(Continued)

	1111111111222222222233333333334444444444555
	12345678901234567890123456789012345678901234567890123456789012
E. misera	00011000200000000000001121110101101001001200020101?1
E. leucocephala	00100001002000011010001121110001101001001201000101?1
E. cotinifolia	00100001002000001010001121110001101001001201020101?1
E. chersonea	21000010100010011000001121110101112??000112011210012?1
E. uniglandulosas	21000000010000010000001121110?011112010012011211012?1
E. graminea	11000000?000001000000112111000111100000120112110 1?1
E. ariensis	11000010001000011010001121110001111000001201120101?1
E. francoana	21000000010000?000000112111010111120100120112010 1?1
E. multiseta	21000000010000010000001121110?011012010012011201012?1
E. ocymoidea	21000000010000000000001121110101111200001201120101 01
E. marginata	21000010100000011010001121110101101001001200021001 01
E. sarcodes	00000010000000?100000112111000110120000120112100101
E. heterophylla	21000000101010001010001121110001110?0201120112100101
E. dentata	2100000010101 00?10100011211100011 10?0201120110100101
E. atoto	11000000012001110000001121110001111000001200020001 11
E. supina	21000000012011110000001121110111111000001201120012001111
E. augusta	11000000012001110000001121110111111000001201120012001111
Tribe Hippomaninae:	
M. brasiliensis	00000000?000000000000000000000001000?0?00000000000000
S. brasiliensis	00000000?000000000000000000000000000?0?00000000000000

Note. "?" designates missing states; classification system based on Webster (1994*b*). Total number of species within the genus is given in parentheses.

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ORDER EUPHORBIALES

An anomalous group, including only the following family. Hutchinson suggests a possible origin from *Malvales* and *Sapindales*. The plants seem to be highly specialized, as evidenced by very reduced flowers in many, the peculiar and flower-like inflorescence in *Euphorbia*, and the milky juice and attendant complex secretory tissue.

EUPHORBIACEAE: Spurge Family

Herbs, shrubs, or trees, often with milky juice. Leaves simple or compound, usually alternate, sometimes reduced to spines. Flowers monoecious or sometimes dioecious, commonly in cymes, with or without a perianth, sometimes with a corolla. Staminate flowers variable, often reduced to a single stamen. Pistillate flowers rather uniform, consisting of a single pistil of mostly 3 carpels, 3-lobed, and forming a capsule or schizocarp which splits into three 1-seeded nutlets in fruit. In specialized forms, such as *Euphorbia*, the inflorescence may simulate a flower, being reduced to one or more cymules or cyathia, these sometimes with ornamental bracts.

The family includes about 283 genera and 7,300 species, widely distributed in temperate and tropical regions, the Indo-Malayan region and Brazil being the chief centers of distribution. Some African species of *Euphorbia* resemble cactus plants. Economically important products include food, drugs, and rubber. Many species are poisonous, the genus *Toxicodendron* of South Africa including some of the most poisonous plants known.

EXAMPLES: *Euphorbia:* the largest genus, with 1,600 species estimated, including *E. pulcherrima*, the Poinsettia.
Hevea braziliensis: an important rubber tree.
Manihot utilissima: Cassava, furnishing tapioca and arrowroot starch from the tuberous roots.
Ricinus communis: Castor Bean, the source of castor oil.

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Distinguishing among *Magnolia* cultivars using fluorescent Amplified Fragment Length Polymorphism (AFLP) analysis

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Abstract Amplified Fragment Length Polymorphism (AFLP) analysis was used to generate informative DNA fingerprints from *Magnolia denudata*, *M. liliiflora*, and putative cultivars of their hybrid cross, i.e., *M.* × *soulangeana*, *M.* × *soulangeana* 'Lennei', *M.* × *soulangeana* 'Rustica Rubra', *M.* × *soulangeana* 'Alba', *M.* × *soulangeana* 'Ruby', and *M.* × *soulangeana* 'San Jose'. A single specimen of unknown origin and representatives of the closely related *M. campbellii* and *M. stellata* were also included in the analysis. We show how this method may be used for distinguishing among cultivars.

Keywords Amplified Fragment Length Polymorphism (AFLP); cultivar; DNA fingerprinting; hybrid; *Magnolia*

INTRODUCTION

A cultivar must be clearly distinct, uniform and stable in its characteristics (Trehane et al. 1995). Distinguishing among cultivars based on morphology can be difficult because the diagnostic characters are often small or may not be apparent all year round. DNA fingerprinting provides a means to test the hybrid origin of cultivars and cultivar similarity and may lead to a better understanding of our cultivated plants (Lee et al. 1996).

Magnolia × *soulangeana* or the saucer magnolia, resulted from a cross between *M. denudata* and *M. liliiflora* made by Etienne Soulange-Bodin in 1820. Soulange-Bodin established and was the first director of the Royal Institute of Horticulture near Paris where he produced this hybrid (Spongberg in Hunt 1998). Although commonly listed as *M.* × *soulangiana*, the correct spelling for this hybrid is with the termination *-eana* as described by Hunt (1998). Callaway (1994), considered it likely that Soulange-Bodin's cross was also made independently in Japanese nurseries, possibly occurring spontaneously when the two parent plants were grown in close proximity. After the introduction of the hybrid by Soulange-Bodin, a plethora of named forms were introduced, including seedlings from backcrosses and open pollinations. These forms cover the complete range between the two parents in flower colour, shape, and size. It is now practically impossible to keep track of all of the forms and account for their origins.

A rootstock of unknown origin in the *Magnolia* collection at Lincoln University, New Zealand that grew after the death of the scion *M. acuminata* 'Golden Glow' produced flowers similar to *M.* × *soulangeana* cultivars in shape and colour, but were generally much larger. Flowering period and morphological characters were insufficient to distinguish this taxon, which has potential as a new cultivar for amenity horticulture.

The DNA fingerprinting technique known as Amplified Fragment Length Polymorphism (AFLP) (Vos et al. 1995; Mueller & Wolfenbarger 1999) may be applied directly to distinguish among and identify cultivars (Baraccia et al. 1998; Dirlewanger et al. 1998; Nadarajan et al. 1999; O'Hanlon et al. 1999; Tignon et al. 2000). The use of DNA profiling for plant variety registration is attracting particular attention (Law et al. 1998). The AFLP method allows the generation of 10–100 times more markers

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per reaction than other fingerprinting techniques, the large number of putative loci sampled providing a measure of variation across a wide portion of the genome (Sharma et al. 1996).

In the present investigation, we used fluorescent AFLP analysis to study genetic variation in *M. denudata* Desr., *M. liliiflora* Desr., and cultivars of their hybrid cross. *M. campbellii* Hook. & Thoms. and *M. stellata* (Sieb. & Zucc.) Maxim. were included for reference because these species are estimated (Qui et al. 1995) to be among the closest relatives of *M. denudata* and *M. liliiflora*. The main question we addressed was: can *Magnolia* cultivars be distinguished using AFLP markers?

MATERIALS AND METHODS

Plant materials and DNA samples

Samples from 12 mature *Magnolia* specimens were used for the present study (Table 1). A single cultivar of unknown origin was included for investigation, plus samples of *M. campbellii* and *M. stellata*. Two leaves were used for separate extractions from *M.* × *soulangeana* and carried through the complete AFLP process as a control. All selective amplifications were repeated for the first primer combination and subsequently carried through as a test of reproducibility.

DNA extraction, digestion, and ligation

Total DNA was extracted using a modification of the CTAB method (Doyle & Doyle 1987). DNA was precipitated in ethanol and resuspended in 25 µl TE (10 m*M* Tris-HCl, pH 8.0; 0.1 m*M* EDTA) buffer. DNA concentrations were estimated and standardised against known concentrations of Lambda DNA on 1% agarose gels.

Initially 10 µl of the total DNA extract was digested with 6 µl digestion buffer (50 m*M* Tris-HCl, pH 7.5; 50 m*M* $(CH_3COO)_2Mg.4H_2O$; 250 m*M* CH_3COOK), 5 µl dH_2O, 1 µl of each restriction enzyme; *Eco*RI (10 U/µl, Roche Molecular Biochemicals) and *Tru*9I (an isoschizomer for *Mse*I) (10 U/µl, Roche Molecular Biochemicals), for 2 h at 37°C followed by 15 min at 70°C. A 5 µl aliquot was run on a 1% agarose gel to check digestion was complete and the remaining DNA was then ligated by adding 1 µl of each of the *Eco*RI (5 pmol/µl) and *Mse*I (50 pmol/µl) adapters (Life Technologies), 2 µl of ligation buffer (100 m*M* Tris-HCl, pH 8.3; 15 m*M* $MgCl_2$; 500 m*M* KCl), 1 µl T4 DNA ligase (1 U/µl, Gibco), and 5 µl dH_2O. The mixture of ligation cocktail plus digested DNA was incubated overnight at 4°C.

Pre-and selective amplifications

The total pre-amplification reaction volume was 40 µl. This reaction consisted of 2 µl ligated DNA (diluted 1:5), 4 µl of 10 × PCR reaction buffer (100 m*M* Tris-HCl, pH 8.3; 15 m*M* $MgCl_2$; 500 m*M* KCl), 0.8 µl 10 m*M* dNTP, 31.04 µl dH_2O, 0.16 µl Taq Polymerase (5 U/µl, Roche Molecular Biochemicals), and 1 µl of each pre-amplification primer, each with one selective nucleotide; *Eco*R1+1 primer 5′–GACTGCGTACCAATTCA–3′ and *Mse*I+1 5′–GATGAGTCCTGAGTAC–3′ (50 ng/µl, Life Technologies). Pre-amplification was carried out in a

Table 1 *Magnolia* species, cultivars, and hybrids, Lincoln University, New Zealand, living plant and herbarium (LINC) voucher numbers for samples used in this study.

Magnolia species/cultivar	Plant no.	Herbarium no.
M. campbellii	None	LINC20004
M. stellata	59	LINC20007
M. denudata	39	LINC20010a
M. liliiflora	62	LINC20008
M. liliiflora 'Nigra'	3	LINC20005
M. × *soulangeana*	27	LINC20009
M. × *soulangeana* 'Lennei'	46	LINC20032
M. × *soulangeana* 'Rustica Rubra'	1	LINC20003
M. × *soulangeana* 'Alba'	14	LINC20035
M. × *soulangeana* 'Ruby'	78	LINC20006
M. × *soulangeana* 'San Jose'	2	LINC20036
M. × "unknown"	18	LINC20002

thermocycler using the following temperature profile: 40 cycles of 30 s at 94°C, 30 s at 50°C, 60 s at 72°C.

The total selective amplification reaction volume was 20 µl and contained 1 µl of preamplification product (diluted 1:10), 2 µl 10 × PCR reaction buffer without $MgCl_2$ (as supplied with Expand High Fidelity PCR system, Roche Molecular Biochemicals), 2.5 µl 25 m*M* $MgCl_2$, 0.5 µl 10 m*M* dNTP, 11.8 µl dH_2O, 0.2 µl Taq Polymererase and 1 µl of each of the selective primers (10 ng/µl EcoRI and 50 ng/µl, MseI, Life Technologies). *Eco*RI primers were labelled with HEX. Selective amplifications were made using four primer combinations: (1) *Eco*R1-AGC/*Mse*I-CAG; (2) *Eco*R1-AGC/*Mse*I-CAT; (3) *Eco*R1-AGC/*Mse*I-CTG; (4) *Eco*R1-ACG/*Mse*I-CAT. The following cycle profile was used for selective amplification: 5 cycles of 30 s at 94°C, 30 s at 65°C, 60 s at 72°C, followed by 6 cycles of 30 s at 94°C, 30 s at 60°C, 60 s at 72°C, followed by 24 cycles of 30 s at 94°C, 30 s at 56°C, 60 s at 72°C.

Generation of AFLP fingerprints

From the 20 µl selective amplification product, 4 µl was run out on a 3% agarose gel to check for distinct amplification products. The remaining 16 µl was purified via ethanol precipitation and aliquots of the products run out on an ABI 377 automatic sequencer. Fragments were detected by laser and accurately sized with an internal standard. Digitally converted raw data were saved as samples migrated past the fluorescence detector. Multilocus profiles were visualised using ABI GENESCAN software. Fragment differences between samples were identified as those differing by 1 bp. AFLP profiles were scored for presence/absence of fragments between 50 and 400 bp and a binary matrix prepared.

Data analyses

The statistical software package SYSTAT vers. 5 was used to calculate the percentage congruence between replicate samples using the simple matching coefficient.

SYSTAT vers. 5 was also used to compare three levels of genetic variation: (1) between replicate selective amplifications; (2) between *M.* × *soulangeana* cultivars; and (3) between species. A similarity matrix was generated for primer combination 1 based on the coefficient of Jaccard (S) (Sneath & Sokal 1973). This coefficient does not consider the shared absence of a character as indicating similarity:

$$S = a / (a + b + c)$$

where a = number of fragments present for both samples; b = number of fragments present for Sample A, but not for Sample B; c = number of fragments present for Sample B, but not for Sample A. When comparing variation between samples, the similarity between every possible pair was used to calculate the median similarity index (Sokal & Rohlf 1981) for each level of variation present among the operational taxonomic units (OTUs) in primer combination 1.

Using the numerical taxonomy program NTSYS-pc (Rohlf 1990), similarity matrices were generated from each of the binary matrices for each primer combination based on Jaccard's coefficient. A pairwise correlation was calculated between the elements of each of these similarity matrices and the Mantel test (Mantel 1967), with 100 random permutations performed to test for significant similarities between primer combinations.

Binary matrices for each primer combination were modified so that each contained only those OTUs shared among all matrices. The matrices with identical OTUs were then pooled to form a single matrix. The coefficient of Jaccard was then used to generate a matrix of similarities for: (1) cluster analysis using the unweighted pair-group method arithmetic average (UPGMA); and (2) principal coordinates analysis (PCOORDA). A UPGMA dendrogram was constructed and the first two coordinates were plotted from the PCOORDA.

The program AFLPapp (Benham, J. 1997: AFLPapp. A tool for analysis of AFLP data: http://hordeum.msu.montana.edu/software/software.html) was used to identify the fragments inherited from each putative parent for all primer combinations. The method takes the first parent and compares every fragment to the second parent. Fragments were not used in the analysis if: (1) a fragment from the first parent is also present in the second parent; and (2) if a fragment was not shared between any of the cultivars and parents. The *Magnolia* of unknown origin was included for analysis.

RESULTS

The congruence between replicate amplifications was 91% (81–100%). Median levels of similarity among OTUs in primer combination 1 based on the Jaccard coefficient were as follows: (1) between replicate selective amplifications, 85%; (2) between

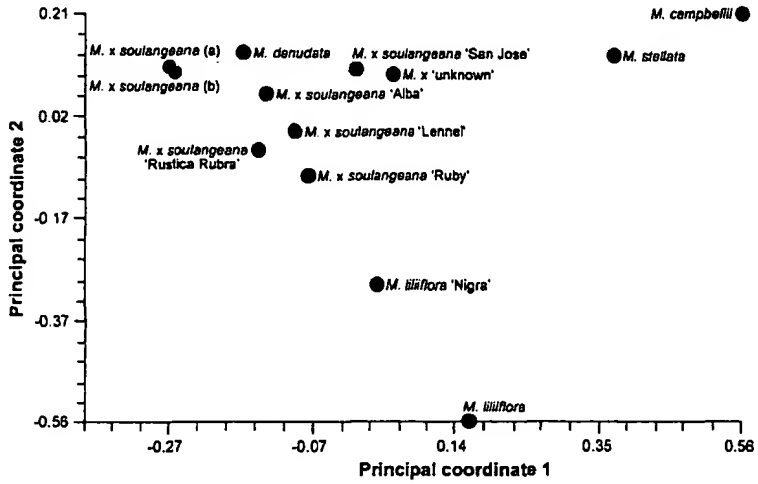

Fig. 1 Plot of principal coordinate 2 versus 1 for *Magnolia* amplified fragment length polymorphisms from four selective primer combinations. *M.* × *soulangeana* (a) and (b) represent different leaves from the same tree.

Table 2 Polymorphism detected with four Amplified Fragment Length Polymorphism (AFLP) primer combinations for 13 *Magnolia* samples.

AFLP primer combination	No. of fragments	No. of polymorphic fragments	% polymorphism
*Eco*R1-AGC/*Mse*I-CAG	283	225	79.5
*Eco*R1-AGC/*Mse*I-CAT	447	328	73.4
*Eco*R1-AGC/*Mse*I-CTG	328	276	84.1
*Eco*R1-ACG/*Mse*I-CAT	343	309	90
Total	1401	1138	81.2

M. × *soulangeana* cultivars, 64%; and (3) between species, 55%.

A total of 1401 AFLP fragments were recorded for 13 OTUs for the four pooled AFLP primer combinations, with an average polymorphism of 81.2% (Table 2). The Mantel test showed a highly significant (P = <0.001) correlation between all combinations of primer matrices. The matrix correlation statistics for each primer combination were: r = 0.62 for primer 1 versus 2, r = 0.69 for primer 1 versus 3, r = 0.69 for primer 1 versus 4, r = 0.85 for primer 2 versus 3, r = 0.86 for primer 2 versus 4, r = 0.86 for primer 3 versus 4. Good separation was achieved using principal coordinates analysis, with the first coordinate explaining 64.2% of the variation. The second and third coordinates explained only 5.1 and 4.3% of the total variation respectively. Principal coordinate axes 1 and 2 (Fig. 1) and UPGMA clustering (Fig. 2) were generally in concordance, both showing that: (1) *M. campbellii* and *M. stellata* were separated from other OTUs; (2) *M.* × *soulangeana* cultivars grouped more closely with *M. denudata* than with *M. liliiflora*; (3) samples originating from separate leaves of *M.* × *soulangeana* grouped closely together; and (4) the sample of unknown hybrid origin appeared among the OTUs representing *M. denudata* and *M.* × *soulangeana* cultivars. No ties were encountered during UPGMA analysis.

Magnolia × *soulangeana* cultivars contained greater proportions of fragments inherited from *M. denudata* compared with *M. liliiflora* (Fig. 3). Numbers of fragments shared between cultivars and parents ranged from *M.* × *soulangeana* 'Alba' (165 fragments shared with *M. denudata* and 73 fragments shared with *M. liliiflora*), to *M.* × *soulangeana* 'Lennei' (139 fragments shared with *M. denudata* and 99 fragments shared with *M. liliiflora*).

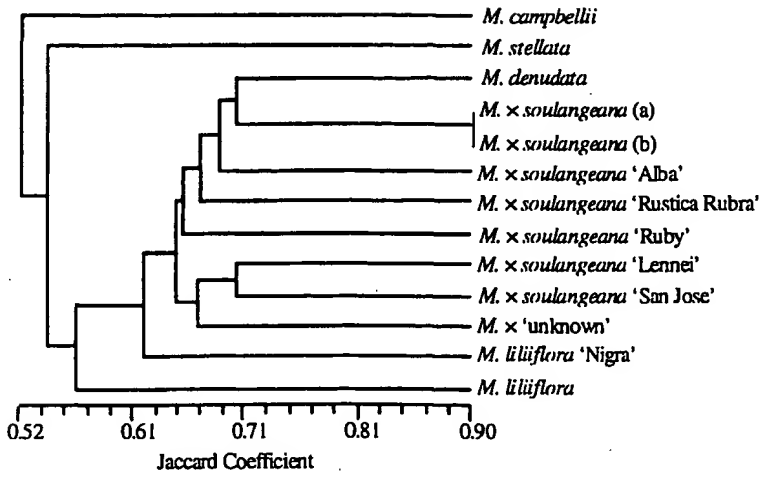

Fig. 2 Unweighted pair-group method arithmetic average (UPGMA) analysis of *Magnolia* amplified fragment length polymorphisms from four selective primer combinations. *M.* × *soulangeana* (a) and (b) represent different leaves from the same tree.

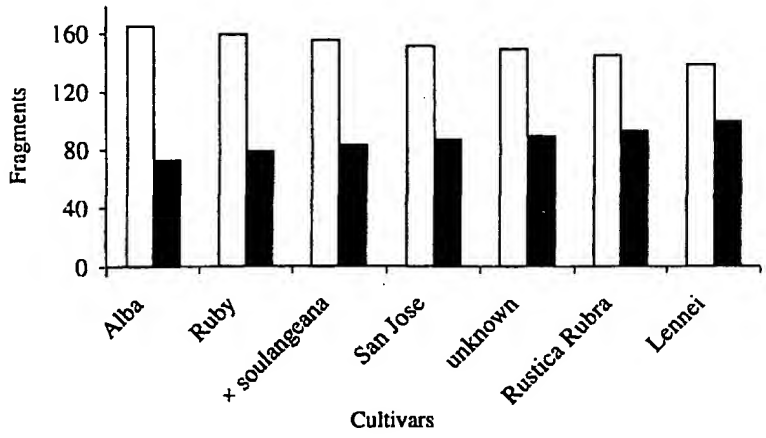

Fig. 3 Number of amplified fragments from four primer combinations shared between *Magnolia* × *soulangeana* cultivars and their parents, *M. denudata* (□) and *M. liliiflora* (■).

DISCUSSION

The results show AFLP data are useful for detecting variation within and among *Magnolia* cultivars. The 91% congruence for replicate AFLP profiles are comparable with previous studies (Huys 1996; Winfield et al. 1998) that reported 95–100% variation for reference samples, indicating the error present is similar to that commonly encountered. The presence of error in the form of non-homologous AFLP products, may bias genetic distance estimates between taxa. Rieseberg (1996) suggested that Random Amplified Polymorphic DNA (RAPD) homology was a function of taxonomic distance, i.e., the more closely the compared taxa are, the greater the probability that a shared co-migrating band is homologous. This might also be extended to the analysis of AFLPs. In the present study, the median similarities based on the Jaccard coefficient were generated specifically for the purpose of comparing different taxonomic levels. These showed decreasing percentage similarity from replicate selective amplifications, to cultivars of *M.* × *soulangeana* and finally samples representing different species.

Magnolia × *soulangeana* cultivars grouped most closely with *M. denudata*, based on PCOORDA and UPGMA clustering (Fig. 1 and 2). All cultivars inherited a higher number of fragments from *M. denudata* compared with *M. liliiflora* (Fig. 3). We were able to estimate that the cultivar of unknown origin included in our study may have originated from a cross between *M. denudata* and *M. liliiflora*, because it was most closely related to the *M.* × *soulangeana* cultivars 'San Jose' and 'Lennei'

(Fig. 1 and 2) and compared with the other cultivars, contained a similar number of fragments inherited from *M. denudata* and *M. liliiflora* (Fig. 3). Unpublished flowering records of magnolias at Lincoln for 3 years from 1988 showed that the unknown magnolia was more similar to *M.* × *soulangeana* 'San Jose' than *M.* × *soulangeana* 'Lennei' in terms of the spring flowering period and accumulated thermal time. But *M.* "unknown" was unlike both *M.* × *soulangeana* 'San Jose' and *M.* × *soulangeana* 'Lennei' in that only these two cultivars of *M.* × *soulangeana* have a second and significant flowering period in late summer. *M.* × *soulangeana* 'Ruby' has also shown some tendency to flower in late summer, but to a lesser extent. A significant period of flowering in late summer was also recorded for *M. liliiflora* and *M. liliiflora* 'Nigra' for each of the 3 years.

Possible explanations for the close relationship implied for *M.* × *soulangeana* cultivars with *M. denudata* include: (1) the specimens do not represent true *M.* × *soulangeana*, and are in fact simple cultivars of *M. denudata*; (2) the specimens are *M.* × *soulangeana*, but segregation has occurred in the F_2 and succeeding generations; and (3) the specimens are *M.* × *soulangeana*, but backcrossing to *M. denudata* has occurred. Spongberg (1998) considered many forms of *M.* × *soulangeana* to approach or merge with one or other parent, possibly as a result of segregation and introgression. Future investigations of *M.* × *soulangeana* hybrid origin should include multiple representatives of each parent, particularly *M. denudata*.

AFLP variation may have potential for profiling *Magnolia* cultivars, providing evidence in addition to morphology, for registration purposes. However, it may be advantageous to apply selection criteria to fragments included for analysis, e.g., Escaravage et al. (1998), who considered only fragments of certain intensity in their analysis of clonal diversity in a *Rhododendron* population. In this way it may be possible to reduce error that might result from scoring artefact fragments that may result from partial digestion of the template genomic DNA, poor amplification of fragments during PCR, etc. Tignon et al. (2000) reported uniformity of AFLP profiles among representatives of most apple cultivars they tested, but one cultivar showed variability between specimens, which indicated to them, a level of genetic instability within this cultivar. This highlights the need for adequate testing of multiple representatives of each cultivar (and also from generation to generation) before the use of AFLP profiling as a tool for registration purposes. Accurate identification of cultivars would also require a database containing DNA fingerprints for all recognised cultivars for comparison.

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Comparative analysis of genetic similarity among maize inbred lines detected by RFLPs, RAPDs, SSRs, and AFLPs

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Abstract DNA-based fingerprinting technologies have proven useful in genetic similarity studies. RFLP is still most commonly used in the estimation of genetic diversity in plant species, but the recently developed PCR-based marker techniques, RAPDs, SSRs and AFLPs, are playing an increasingly important role in these investigations. Using a set of 33 maize inbred lines we report on a comparison of techniques to evaluate their informativeness and applicability for the study of genetic diversity. The four assays differed in the amount of polymorphism detected. The information content, measured by the expected heterozygosity and the average number of alleles, was higher for SSRs, while the lowest level of polymorphism was obtained with AFLPs. However, AFLPs were the most efficient marker system because of their capacity to reveal several bands in a single amplification. In fact, the assay efficiency index was more than ten-fold higher for AFLPs compared to the other methods. Except for RAPDs, the genetic similarity trees were highly correlated. SSR and AFLP technologies can replace RFLP marker in genetic similarity studies because of their comparable accuracy in genotyping inbred lines selected by pedigree. Bootstrap analysis revealed that, in the set of lines analysed, the number of markers used was sufficient for a reliable estimation of genetic similarity and for a meaningful comparison of marker technologies.

Key words *Zea mays* L. · Genetic relationship · Molecular markers · DNA-fingerprinting · Genetic diversity

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Introduction

Knowledge of germplasm diversity and of relationships among elite breeding materials has a significant impact on the improvement of crop plants (Hallauer et al. 1988). In maize, this information is useful in planning crosses for hybrid and line development, in assigning lines to heterotic groups, and in plant variety protection. It can be obtained from pedigree and heterosis data, from morphological traits or using molecular markers which detect variation at the DNA sequence level (Smith and Smith 1992). In particular, DNA-based polymorphisms are a powerful tool in the assessment of the genetic similarity between breeding stocks (reviewed in Lee 1995).

The discrimination power of restriction fragment length polymorphisms (RFLPs) has been extensively studied in maize, as has their use in establishing relationships with yield and heterosis (Melchinger 1993). However, there are several drawbacks to RFLPs that have stimulated the development of alternative marker systems: large quantities of DNA are in fact required for RFLP analysis, which is costly, and the technique is difficult to automate. Moreover, it requires sizeable laboratories and specialised equipment.

Various PCR-based marker techniques have recently been successfully introduced in the fingerprinting of plant genomes (Welsh and McClelland 1990; Kesseli

et al. 1994) and in genetic diversity studies (Tinker et al. 1993). Among them, random amplified polymorphic DNA (RAPD) analysis is quick (Welsh and McClelland 1990; Williams et al. 1990) and well adapted for the efficient non-radioactive DNA fingerprinting of genotypes (dos Santos et al. 1994; Thormann et al. 1994). Problems with the reproducibility of amplification and with the scoring of error data have been reported for RAPDs (Demeke et al. 1997; Karp et al. 1997).

Eukaryotic genomes are interspersed with tandem repeats of DNA, referred to as microsatellites or simple sequence repeats (SSRs). SSR polymorphisms have been extensively used as genetic markers in mammals (Tautz 1989); they occur frequently also in plant genomes, showing an extensive variation in different individuals and accessions (Akkaya et al. 1992; Senior and Heun 1993; Wu and Tanksley 1993). SSR loci are co-dominant markers more informative than RAPDs and RFLPs (Russell et al. 1997). Specific technical developments are underway (Mitchell et al. 1997) that should result in the provision of SSRs that will be faster, more standardised and more effective than RFLP technology.

Amplified fragment length polymorphism (AFLP™) is a multilocus marker technique developed by Vos et al. (1995). AFLP markers are genomic fragments detected after selective PCR amplification which provide a number of appealing features in the fingerprinting of genomes of different complexity, including that of maize (Vos et al. 1995). The AFLP technique has been used to identify markers linked to disease resistance loci (Becker et al. 1995; Cervera et al. 1996), to fingerprint DNAs (Vos et al. 1995; Sharma et al. 1996), and to assess relationships between molecular polymorphism and hybrid performance in maize (Ajmone-Marsan et al. 1998).

A comparison of different marker techniques is timely, even though the utility of different molecular markers for soybean and barley germplasm has already been reported (Powell et al. 1996; Russell et al. 1997). The objectives of the present study were: (1) to compare the informativeness of different molecular markers and their applicability for genetic diversity analysis, genotype identification and variety protection purposes, (2) to determine the genetic similarity obtained with RFLP- and PCR-based techniques in a set of maize inbred lines, and (3) to compare their effectiveness in estimating genetic similarity among maize inbreds.

Materials and methods

Plant materials and DNA extraction

Thirty three inbred lines were chosen to explore the diversity of maize germplasm. All these inbreds have been extensively used in the production of hybrid seed and in maize breeding programs. Pedigree information was previously described in Livini et al. (1992). Based on available information and on the heterotic behaviour in crosses, 13 (A641, B14 A, B37, B73, B84, Cm109 Lo1016, Lo916, Lo950, Lo951, Lo964, Lo999, and N28) can be associated with the Iowa Stiff Stalk Synthetic (BSSS) heterotic group, 13 (A619, C103, C123, H99, Lo881, Lo924, Lo976, Lo1077, Mo17, Oh43, Va22, Va59, and Va85) with the Lancaster Sure Crop (LSC), two to Wf9 (WF9 and Pa91), three to W153R (W153R, Lo932, and Lo944), and two to HY (H55 and H96). Genomic DNA was isolated from a bulk of 20–30 shoots of 7–9-day old germinated seedlings and extracted using the CTAB method as previously described (Livini et al. 1992).

Nucleic-acid manipulation and molecular-marker assays

Conditions for restriction enzyme digestion, gel electrophoresis for RFLP, Southern transfer hybridisation, and autoradiography followed Livini et al. (1992). Forty seven genomic clones from the UMC and BNL collections and two restriction enzymes (*Eco*RI and *Hin*dIII) were used to characterise 53 RFLP loci in the 33 inbred lines. A total of 253 RFLP bands were binary coded as 1 or 0 for the presence or absence of such loci in each line, respectively.

RAPD amplification was performed as described by Ajmone-Marsan et al. (1993) using a Perkin Elmer 9600 Thermal Cycler. Reaction products were analysed by electrophoresis on a 2% agarose gel and stained with ethidium bromide. A total of 85 primers (Operon Technologies, California, USA) were surveyed in two inbred lines. Twenty five primers showing reproducible, and clearly scorable, polymorphic (present or absent) fragments, were used to fingerprint the 33 inbreds.

The primers for the SSR markers were synthesised according to the sequences published by Senior and Heun (1993) and Taramino and Tingey (1996). SSR procedures were those described by Taramino and Tingey (1996). Processed fragments, along with loading dye and internal size standards, were run out on a 6% acrylamide gel (Pfeiffer et al. 1997) using an Automated Laser Fluorescent sequencing electrophoresis unit (Pharmacia). Fragments were labelled with fluorescein by direct incorporation of F-12-dUTP (2-μM final concentration) during the PCR reaction. Un-incorporated labelled nucleotide was removed by ethanol precipitation prior to loading samples on the gel. Data were processed using Fragment Manager Software v. 1.1 (Pharmacia). SSR bands were sized first and then binary coded by 1 or 0 for their presence or absence in each line.

AFLP marker analysis was according to Vos et al. (1995). Briefly, total genomic DNA (400 ng) was restricted with 5 U of *Eco*RI (rare cutter) and 5 U of *Mse*I (frequent cutter) (Pharmacia), and double-stranded adapters ligated to the fragment ends. The structure of the adapter sequences, pre-amplification, amplification and polyacrylamide-gel electrophoresis conditions were as in Ajmone-Marsan et al. (1998). Polymorphic amplification products were visualised by autoradiography and scored manually. All AFLP polymorphisms were scored as dominant markers.

All names of the RFLP probes and the nucleotide sequences of the primers used for the amplification of AFLP, RAPD, and SSR markers are available on request.

Data analysis

The average number of alleles per locus, the allele frequency, the expected heterozygosity (He), and the effective number of alleles per locus were calculated as reported by Morgante et al. (1994). The total number of effective alleles (Ne) surveyed by RFLP, RAPD, SSR, and AFLP analyses was calculated by summing the number of effective alleles of all the analysed loci as $Ne = \sum ne\,(i)$. To compare the efficiency among the four methods, where RFLPs and SSRs generally detect multiple alleles and one band per assay, whereas RAPDs and AFLPs detect two alleles and multiple bands per assay,

an assay efficiency index (Ai) was calculated. Ai combines the effective number of alleles identified per locus and the number of the polymorphic bands detected in each assay as Ai = Ne/P, where Ne is the total number of effective alleles detected and P is the total number of assays performed for their detection.

The genetic similarities (GSs) from RFLP, RAPD, SSR, and AFLP data were calculated among all possible pairs of lines using the Dice similarity index as in Nei and Li (1979). The co-ancestry coefficient, *f*, between lines related by pedigree, was calculated as previously reported (Ajmone-Marsan et al. 1992). Cluster analyses were based on similarity matrices obtained with the unweighted pair group method using arithmetic averages (UPGMA) (Rohlf 1990) and relationships between inbred lines were visualised as dendrograms. For each dendrogram the co-phenetic coefficients between the matrix of genetic similarities and the matrix of co-phenetic values were computed using appropriate routines of the NTSYS-pc package. The significance of the co-phenetic correlation observed was tested using the Mantel matrix correspondence test (Mantel 1967).

The bootstrap procedure was employed to determine the sampling variance of the genetic similarities calculated from the data sets obtained with the different marker systems. All data, irrespective of the dual or multiallelic nature of the marker system, were scored in the form of a binary matrix. For each pair of inbreds, the Dice similarity index (GS) was calculated from the 2000 random subsamples at different sample sizes (10, 50, 100, 150, 200, and all bands when the total exceeded 200). Bootstrap standard deviation estimates were based on 2000 samples. The calculations were performed with the SAS macro "BOOT" (Jackknife and Bootstrap Analyses, SAS Institute Inc.).

Results

Levels of polymorphisms

The 33 inbred lines were surveyed with the four different marker systems. All of the molecular markers were able to uniquely fingerprint each of the inbred lines. The levels of polymorphism detected with each marker system and the index comparing their informativeness are reported in Table 1. The total number of assays ranged from only six primer combinations for AFLPs to 53 probe/enzyme combinations for RFLPs. The total number of polymorphic bands identified ranged from 90 for RAPDs to 253 for RFLPs. An average number of 4.8 alleles per locus, with an average effective number of 3.2 alleles per locus, ranging from 1.2 to 6.5, could be distinguished for each probe/enzyme combination using RFLPs. This value increased to 6.8 with SSRs, with an average number of effective alleles of 4.4 per locus, ranging from 1.1 to 6.6, while for RAPDs and AFLPs these values were lower (1.6 for both). This was reflected also in lower expected heterozygosity values. Overall the highest assay efficiency index was observed for AFLPs (61.9) and the lowest for RFLPs (3.2). RAPDs and SSRs (5.8 and 4.4, respectively) were comparable to RFLPs. In particular, for AFLPs the high assay efficiency index is due to the simultaneous detection of several polymorphic bands in a multiplex amplification per single reaction.

Genetic similarity

A summary of the genetic similarity estimates, calculated for each marker system, between pairs of lines of the various heterotic groups is shown in Table 2. All marker systems indicated that lines of BSSS origin were more similar in comparison to inbred lines of other heterotic groups. The mean value of the GS estimate was, as expected, lower for BSSS × LSC crosses than within the BSSS and LSC groups themselves. This is consistent with the common practice in maize breeding of preferentially developing hybrids between heterotic groups because they are expected to perform better than those from crosses within heterotic groups. The similarity ranged from 0.92 within LSC types, using AFLPs, to 0.00 within BSSS × miscellaneous types, using SSRs. The estimates of GS follow the same pattern across marker systems, i.e. higher estimates of similarity within the BSSS types and lower estimates within the LSC × miscellaneous types. Overall SSRs revealed the lowest similarity values and AFLPs the highest.

The genetic similarity trees produced from each marker system are presented in Fig. 1. In these trees inbreds were ordered as expected, though with exceptions, into the major groups BSSS and LSC.

Table 1 Level of polymorphism and comparison of informativeness obtained with RFLP, RAPD, SSR and AFLP markers in 33 maize inbred lines

Parameters	Marker system			
	RFLP	RAPD	SSR	AFLP
Number of assay units	53 (probe/enzymes)	25 (primers)	27 (primer pairs)	6 (primer combination)
Number of polymorphic bands	253	90	183	232
Number of loci	53	90[a]	27	232[a]
Average number of alleles per locus	4.8	2.0	6.8	2.0
Expected heterozygosity	0.63	0.36	0.72	0.34
Effective number of alleles per locus	3.2	1.6	4.4	1.6
Assay efficiency index	3.2	5.8	4.4	61.9

[a] Theoretical maximum number of loci

Table 2 Mean, minimum and maximum of the Dice genetic similarity coefficient (GS) calculated from different molecular marker systems for various groups of maize inbred lines

Marker system		GS value						
		Within heterotic groups			Between heterotic groups			Between all lines
		BSSS ($n = 78$)	LSC ($n = 78$)	MISC ($n = 21$)	BSSS × LSC ($n = 169$)	BSSS × MISC ($n = 91$)	LSC × MISC ($n = 91$)	ALL ($n = 528$)
RFLP	Min.	0.28	0.25	0.23	0.18	0.20	0.18	0.18
	Max.	0.79	0.86	0.90	0.59	0.49	0.56	0.90
	Mean	0.48	0.40	0.41	0.36	0.36	0.35	0.37
RAPD	Min.	0.34	0.38	0.31	0.37	0.36	0.29	0.29
	Max.	0.90	0.80	0.89	0.76	0.65	0.68	0.91
	Mean	0.64	0.57	0.53	0.58	0.53	0.53	0.56
SSR	Min.	0.13	0.08	0.08	0.07	0.00	0.04	0.00
	Max.	0.84	0.88	0.82	0.52	0.52	0.40	0.88
	Mean	0.38	0.31	0.28	0.27	0.22	0.20	0.26
AFLP	Min.	0.45	0.43	0.43	0.36	0.43	0.39	0.36
	Max.	0.89	0.92	0.89	0.61	0.64	0.62	0.92
	Mean	0.62	0.59	0.55	0.48	0.52	0.51	0.53

Discrepancies in forming subgroups within the major groups were observed as well as in the clustering of inbred lines of miscellaneous origins. Considering the BSSS-related lines the topology of each tree is unique with some evident similarity: the clustering of B14, B37, and B73 is, for example, fully conserved. On the LSC side, clustering was consistently reported by all methods with the exception of Va22, a line derived from C103, indicating that all methods aggregated lines of different origin. The Oh43-related lines (Oh43 and A619) were positioned within the Lancaster group only by RAPDs and AFLPs, while SSRs and RFLPs clustered these with BSSS lines (although Oh43 is usually considered a Lancaster type). Similarly to the RAPD-based tree, the clustering based on AFLP data produced a tree with a relatively narrow range of similarity values between the more-related and the more-distant pairs of inbreds. In spite of this, all the main clusters were confirmed by AFLP data.

Four pairs of very similar inbreds (B14 A, Cm109; Lo932, Lo944; A619, Oh43; and H55, H96) were clustered together by all marker systems, while in one additional case similar lines (Lo916 and Lo999) were consistently grouped in AFLP, SSR and RAPD trees.

Comparison between marker systems

All the estimates of correlation coefficients (*r*s) among available co-ancestry coefficients (*f*s) and genetic similarity (GS) data were highly significant ($P < 0.01$). RAPDs showed the lowest correlation ($r = 0.40$) with *f* values, RFLPs and SSRs intermediate values ($r = 0.57$ and $r = 0.53$, respectively), while AFLPs showed the highest value ($r = 0.62$). The *r*s among similarity data were also significant. Correlation coefficients of RAPD marker data ($r = 0.51$, $r = 0.57$, and $r = 0.52$ with RFLP, SSR and AFLP, respectively) with those obtained using other marker systems were lower than those among similarity estimates based on AFLPs, RFLPs, and SSRs ($r = 0.70$, $r = 0.67$ and $r = 0.59$, respectively between AFLP and RFLP, AFLP and SSR, and RFLP and SSR). The extent to which similarities were correlated varied considerably across the whole data set. When the set of pairwise data (528) was divided into two groups (according to the arithmetic mean of the observed GS range based on RFLP data: "more similar" lines with GS > 0.37 and "less similar" lines with GS < 0.37), it became evident that the genetic similarities estimated by different marker systems were mainly correlated only among similar lines, while the relationships among dissimilar lines were low and not significant. The GS values plotted against the estimate of co-ancestry value based on pedigree data followed the same pattern.

The co-phenetic correlation coefficients provided for each marker system indicate the extent to which the clustering of genotypes depicted in the trees accurately represents the estimates of genetic similarity between inbreds obtained with that marker system. Overall the co-phenetic coefficients were medium to high, with the RFLP (0.84) and AFLP (0.83) data resulting in the highest correlation, SSR (0.80) showing an intermediate value, and the RAPD (0.72) assay producing the lowest correlation.

All methods could clearly distinguish all 33 inbred lines, although the SSR data provided the highest level of discrimination between any pair of inbreds. In general, the grouping agreed with the pedigree information of the lines, although some discrepancies were

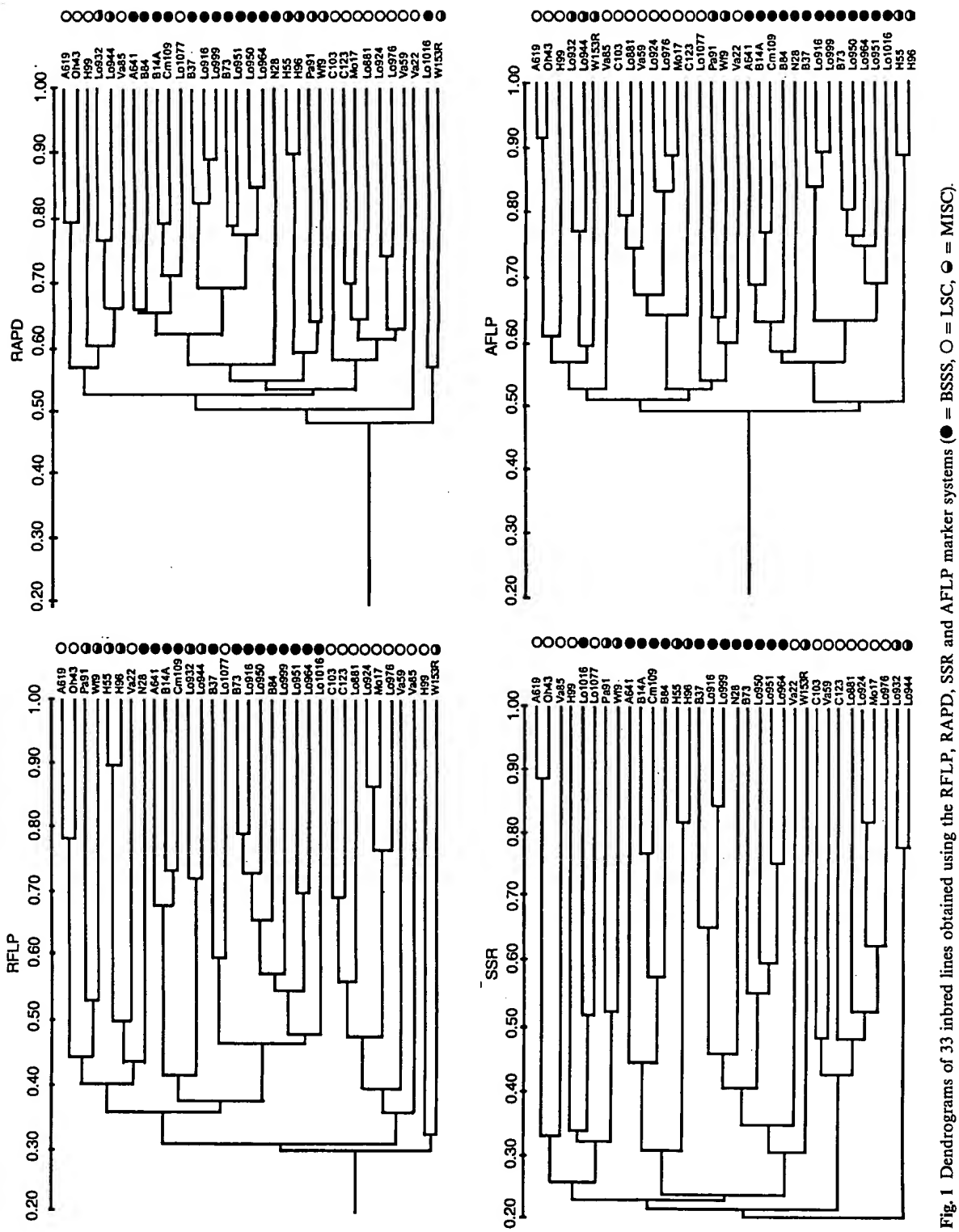

Fig. 1 Dendrograms of 33 inbred lines obtained using the RFLP, RAPD, SSR and AFLP marker systems (● = BSSS, ○ = LSC, ◒ = MISC).

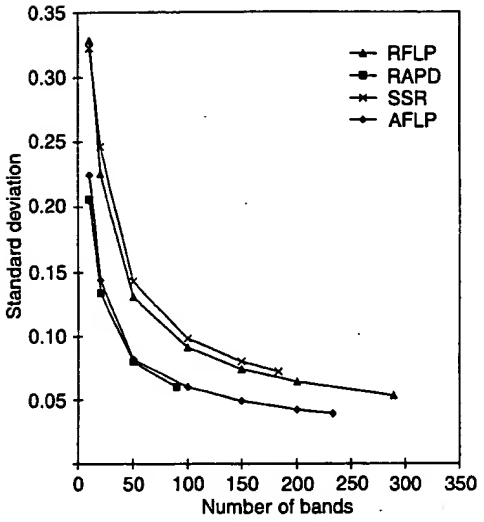

Fig. 2 Variation assessed by bootstrap sampling of genetic similarity between maize inbreds across different marker systems due to the different number of markers (bands)

observed. In particular, genetic similarities based on AFLP data had the highest correlation with pedigree data, while those based on RAPDs had the lowest one.

Bootstrap analysis

To determine the sampling variance of genetic similarities calculated from different molecular marker data sets, bootstrap analysis with a declining number of bands was performed. The relationships between number of bands and the sampling variance of the genetic similarity among all pairs of inbred lines for each method is presented in Fig. 2. The results indicated that above 150 bands there was a diminishing return in the precision gained by adding additional bands. As the number of bands moves below these thresholds the standard deviation begins to increase (and precision decreases) at a greater rate.

Discussion

In this paper we have shown that the number of alleles detectable in maize by SSRs is higher in comparison to other methods. This high level of polymorphism is to be expected because of the unique mechanism responsible for generating SSR allelic diversity by replication slippage (Tautz et al. 1986). It is also known that when SSRs have been compared to other marker systems they have revealed the highest level of polymorphism (Wu and Tanskley 1993; Morgante et al. 1994; Powell et al. 1996). The present data indicate that on average SSRs carry two-fold more information than AFLPs and RAPDs, and 40% more information than RFLPs, when the number of alleles per locus is the target.

In agreement with previous observations (Becker et al. 1995), the lowest degree of polymorphism was associated with AFLPs. Conversely, the information measured as the assay efficiency index, which correlates with the number of effective alleles identified per assay, was more than ten-fold higher for AFLPs compared to the other methods. These findings are in good agreement with previous germplasm analysis carried out in several crop species (Lu et al. 1996; Powell et al. 1996). It can be concluded that SSRs are capable of revealing the highest level of information per single marker and that AFLPs detect the highest number of polymorphisms in a single assay. This high assay efficiency index is a reflection of the efficiency of AFLPs to simultaneously analyse a large number of bands rather than the levels of polymorphism detected at each locus. The assay efficiency index for SSRs can, however, be considerably higher if multiplex PCR and gel-running procedures are adopted, where several microsatellites are simultaneously amplified and co-electrophoresed using multicolour fluorescent technologies (Lindqvist et al. 1996; Heyen et al. 1997). An additional advantage of SSRs over AFLPs is only relevant when mapping populations are derived from outcrossing heterozygous individuals, where the multi-allelism of SSR markers increases the number of informative genotypic classes in the progenies compared to the binary AFLP markers.

The results shown by genetic similarity trees indicate that, except for RAPDs, they are highly similar. In addition, trees from these molecular methods agree with the information obtained from pedigree data. Similarly, Powell et al. (1996) found the lowest correlations among RAPDs and other marker systems. In this respect, it has been shown that RAPD analysis, based on the use of random primers, is likely to suffer from a lack of reproducibility due to mismatch annealing (Neale and Harry 1994). In the trees obtained from cluster analysis, all lines with defined affiliation to one of the heterotic groups were assigned to their specific main clusters, in agreement with the available data for maize (Ajmone-Marsan et al. 1992; Livini et al. 1992; Mumm and Dudley 1994; Smith et al. 1997). A second observation is that, within the clusters, the grouping of more distantly related lines does not match precisely with the expectations based upon pedigree data. Differences among marker techniques in grouping genetically more distant lines have been previously reported (Powell et al. 1996). Other studies in *Brassica* and from pea accessions show that molecular marker-based similarities and trees were significantly correlated across a wide range of germplasms (Thormann et al. 1994; Lu et al. 1996). Many potential reasons for these discrepancies exist, including underlying assumptions in calculating pedigree data (Messmer et al. 1993), genome sampling

(Nei 1987), and the numbers of markers or probes employed (Tivang et al. 1994).

The number of loci required for a reliable estimate of genetic similarity has been shown to vary from 15 RFLP probes, giving 56 bands in *Brassica* sp. (dos Santos et al. 1994), to 100 RFLP clone-enzyme combinations (Messmer et al. 1993). Similarly, Tivang et al. (1994), investigating in maize the sampling variance of a RFLP data set in maize, found that the number of bands required for a CV of 10% was 388, 150, and 38 for closely, intermediately, and distantly related inbreds, respectively. Our results using the bootstrap procedure suggest that 150 bands are sufficient for reliable estimates of genetic similarity. Accordingly, the average number of assays that could have been used in this study to attain such a precision in the estimate were 30–40 clone-enzyme combinations for RFLPs, 40–50 primers for RAPDs, 20–30 primers for SSRs, and 4–5 enzyme combinations for AFLPs. Based on these estimates, the disagreement of the RAPD results in comparison to the other types of markers might be explained by the insufficient number of primers used.

In conclusion, the results of this study indicate that, with the exception of RAPDs, the other DNA markers provide consistent information for germplasm identification and pedigree validation. We have shown that SSR and AFLP profiling technologies can be good candidates to replace RFLP markers in genetic similarity estimates and variety description, and that they have comparable accuracy in grouping inbred lines selected by pedigree. They are generally much simpler to apply and more sensitive than the traditional morphological and biochemical methods or the RFLP-based fingerprinting techniques; yet they provide results correlated with those from RFLP analyses. A major advantage of the SSR and AFLP methods is that they can be automated. While SSRs, thanks to their multi-allelism and co-dominance, appear to be suited for the analysis of outcrossing heterozygous individuals, AFLPs, with their high multiplex ratio, offer a distinctive advantage when genome coverage is a major issue due to the presence of linkage disequilibrium, such as in inbred lines and breeding materials.

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Direct comparison of levels of genetic variation among barley accessions detected by RFLPs, AFLPs, SSRs and RAPDs

Abstract RFLPs, AFLPs, RAPDs and SSRs were used to determine the genetic relationships among 18 cultivated barley accessions and the results compared to pedigree relationships where these were available. All of the approaches were able to uniquely fingerprint each of the accessions. The four assays differed in the amount of polymorphism detected. For example, all 13 SSR primers were polymorphic, with an average of 5.7 alleles per primer set, while nearly 54% of the fragments generated using AFLPs were monomorphic. The highest diversity index was observed for AFLPs (0.937) and the lowest for RFLP (0.322). Principal co-ordinate analysis (PCoA) clearly separated the spring types from the winter types using RFLP and AFLP data with the two-row winter types forming an intermediate group. Only a small group of spring types clustered together using SSR data with the two-row and six-row winter varieties more widely dispersed. Direct comparisons between genetic similarity (GS) estimates revealed by each of the assays were measured by a number of approaches. Spearman rank correlation ranked over 70% of the pairwise comparisons between AFLPs and RFLPs in the same order. SSRs had the lowest values when compared to the other three assays. These results are discussed in terms of the choice of appropriate technology for different aspects of germplasm evaluation.

Key words Barley · Genetic relationships · Molecular analysis · RFLP · AFLP · RAPD · SSR

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Introduction

As one of the first crop plants to be domesticated, barley (*Hordeum vulgare* L.) remains one of the most important crops today. Ranking fourth in world acreage, barley is used for human consumption, as a fodder crop and as a raw material for brewing beer and whisky (Brown 1992). It belongs to the genus *Hordeum*, which comprises over 32 species, including diploid and polyploid, perennial and annual types, which are spread throughout the world. The genus can be divided into three groups of varying importance to cultivated barley improvement; the primary genepool (*H. vulgare* spp. *vulgare* and *H. vulgare* spp. *spontaneum*), the secondary genepool (*H. bulbosum*) and the tertiary genepool (all other *Hordeum* species). Presently more than 250,000 *Hordeum* accessions are held in genebanks throughout the world, and the number is increasing (IBPGR 1992). With the growth of the germplasm collection a need for procedures which will allow their more effective use is required. The 'Core Collection' concept, is one such method, which should provide users with a limited set of genetically distinct and representative accessions (Brown 1989). Recently this concept has been applied to barley, and the Barley Core Collection (BCC), consisting of a limited sample of accessions considered to represent the spectrum of genetic diversity available in the genus, was established (Hintum 1992).

In such collections, morphological data are the principle descriptors which have been used to detail the accessions held. With the development of molecular markers and their many perceived advantages, it is crucial that these techniques are applied to assess genetic diversity in germplasm collections in order to supplement and refine the morphological-based classification. However, in recent years, the number of molecular assays available for application in this area has increased dramatically, with each method differing in principle, in application, in the type and amount of polymorphism detected and in cost and time

requirements. The approaches include restriction fragment length polymorphism (RFLPs; Botstein et al. 1980), random amplified polymorphic DNA (RAPDs; Williams et al. 1990), simple sequence repeat polymorphisms or microsatellites (SSRs; Tautz 1989) and Amplified Fragment Length Polymorphism (AFLPs; Zabeau and Vos 1993).

Faced with this wealth of marker technology, it is appropriate to determine if the same patterns of variability are revealed by each and whether the observed molecular diversity reflects either co-ancestry or morphological classification. To address this we have evaluated and compared similarity measures obtained from the four above systems on a set of accessions which are representative of cultivated European barley germplasm. This has allowed us to compare the results obtained from molecular analysis with each other and with pedigree information. The results are discussed in relation to the overall genetic diversity observed and the features of the individual assays.

Materials and methods

Plant material and DNA isolation

Eighteen accessions (Table 1), representing the majority of ancestors European cultivated barley, were selected for this study. Total genomic DNA was isolated from fresh leaf material by a modification of the method described by Saghai-Maroof et al. (1984).

Marker analysis

RAPD

RAPD amplifications were performed as described by Barua et al. (1993). Fragments were separated on 1.5% agarose gels, stained with ethidium bromide, visualised with ultraviolet light and photographed. The presence or absence of polymorphic bands were scored. Twenty primers, which were polymorphic between the parents of a spring × spring cross ('Blenheim' and E224/3), were used in this study.

RFLP

RFLP profiles were detected according to the protocol described by Graner et al. (1991). DNA was digested with three restriction enzymes (*Bam*HI, *Eco*RI and *Hin*dIII), and restriction fragments were detected using 48 single-copy DNA clones selected from previous mapping experiments to give good genome coverage and levels of polymorphism (Graner et al. 1991). RFLP patterns were scored as presence or absence of bands.

SSR

Two sources of simple sequence repeats were used in this study: database-derived repeats and repeats derived from an enriched genomic library. The 6 database-derived SSRs are described in a recent publication by Becker and Heun (1995). The 7 library-derived SSRs are described by Macaulay et al. (in preparation). SSR assays were performed as described by Morgante et al. (1994). Allele lengths were determined by comparing the most intense band with an M13 DNA sequence marker.

Table 1 Pedigree information and country of origin of 18 barley accessions used in molecular analysis

Cultivar	Pedigree	Origin[a]
Spring type, two-rowed:		
Aramir	Volla × Emir	NL
Beka	Bethge XIII × Kniefel	F
Golden Promise	X-ray mutant from Maythorpe (Irish Goldthorpe × Maja)	GB
Grit	Langenstein-Nungesser (5547/67 × 46459/68) 480/68 or Hadml. 554-Emir-11191-Union-46495-Diamant 14008	D
Hora	Sultan × (Weihenstephaner 1206 Nacktgerste × Volla)	D
Krona	Complex cross including Triumph	D
Triumph	(Hadm.24566 × Diamant × 1402964/6) × ((Alsa × Abyssinian) × St. × Union)	D
Union	(Weihenstephaner Mehtauresistente II × Donaria) × Firlbecks III	D
Volga	Complex cross with eight varieties	F
Winter type, two-rowed:		
Igri	(Malta × Carlsberg 1427) × Ingrid	D
Marinka	(Alpha × SVP 674) × Malta	NL
Romanze	Weihenstephan 4622/73 × (Malta × Sonja)	D
Sonja	Tria × Malta	D
Winter type, six-rowed:		
Borwinia	Vogelsanger Gold × St. 7246	D
Express	Robur × Athene	D
Franka	(Vogelsanger Gold × Senta) × (Dura × Dea) × Vogelsanger Gold	D
Gaulois	Gerbel × Athene	F
Rondo	Tanaroo × Sisfor L. 90	I

[a] NL, The Netherlands; F, France; GB, Great Britain; D, Germany; I, Italy

AFLP

AFLP analysis was essentially as described by Vos et al. (1995). Briefly, 500 ng of genomic DNA was digested with *Eco*RI and *Mse*I and double-stranded adaptors ligated to the fragment ends. This was followed by a pre-amplification step using non-selective primers. Selective amplifications were performed on the pre-amplified fragment mixture using a total of six primer combinations. Only the *Eco*RI primer was radiolabelled with γ-[^{33}P] ATP (ICN), and all primers had three selective nucleotides. Amplification products were separated by denaturing 6% polyacrylamide gel electrophoresis (PAGE), visualised by autoradiography and manually scored for the presence or absence of bands.

All of the primer names and sequences used are available on request from the authors.

Data analysis

Diversity values were calculated for each locus as $(1 - \Sigma\ Pi^2$, where Pi is the phenotypic frequency for each assay unit (RFLPs-probe/enzyme combinations; RAPDs-primers; SSRs-primer pairs; AFLPs-primer combinations). Genetic similarities (GS) were calculated using the GENSTAT Version 531 software package according to Nei and Li's (1979) estimate of similarity. Similarities were expressed using the group average agglomerative clustering function of GENSTAT to generate principal co-ordinate plots (Kempton and McNicol 1990). Correlations between assays were calculated using [Procrustes rotational analysis (PR) on the principal co-ordinate data] Spearman rank correlation (SRC) and linear regression of the GS values.

Results

Fingerprinting

All of the molecular approaches used in this study were able to uniquely fingerprint each of the 18 cultivated barley accessions. The total number of assay units varied for each marker system from only 6 primer combinations for AFLPs to 144 probe/enzyme combinations for RFLPs (Table 2). Similarly, the number of bands scored ranged from 70 for SSRs to 299 for RFLPs. The percentage of polymorphic bands for each assay did not correlate to the total number of bands. For example, only 70 bands were scored for SSRs, which was the lowest number, but all 70 were polymorphic. In contrast, 297 AFLP bands were scored, and only 46.8% of those were polymorphic. RFLPs and RAPDs were intermediate with 83.2% and 66.3%, respectively, of all bands scored being polymorphic. There was wide variation in the average number of genotypes revealed by each marker system (Fig. 1). With RFLPs, for each probe/enzyme combination, an average of 2.37 genotypic classes could be distinguished. With AFLPs this figure increased to 17.2 as nearly all primer combinations were able to discriminate between the 18 accessions used. This is further reflected in the diversity index measures. Overall the highest diversity index was observed for AFLPs (0.937), and the lowest for RFLPs (0.322). RAPDs and SSRs were intermediate (0.521 and 0.566, respectively).

Genetic similarity

The cultivated barley genepool can be divided into spring and winter types. The winter barleys are mainly used for fodder and can be further divided into two- and six-rowed types. The spring barleys are mainly used for malting. The maximum, minimum and mean similarity estimates between the spring barleys and two-row and six-row winter barleys for each assay system are shown Table 3. The similarities ranged from 0.97 within spring types using AFLPs to 0.45 within six-row winter types using SSRs. Between assay systems the estimates of similarity followed the same pattern, i.e. higher estimates of similarity within the spring types (means: RFLPs = 0.843, AFLPs = 0.924, SSRs = 0.829) and lower estimates within the six-row winter types (means: RFLPs = 0.70, AFLPs = 0.877, SSRs = 0.657). Estimates with two-row winter types were intermediate. The situation with RAPDs was different, with spring and six-row winter types exhibiting equivalent mean similarities (0.879 and 0.897, respectively). Overall, SSRs revealed the lowest similarity values (0.93–0.45) and AFLPs the highest (0.97–0.81).

Some accessions can be traced to common ancestors. For example, Grit and Triumph have Union in their pedigrees and Krona has Triumph. Our expectation would therefore be that these 4 accessions should be closely related. Table 4 shows the genetic similarity

Table 2 Analysis of the RFLP-, RAPD-, SSRs- and AFLP-generated banding patterns

Marker	Number of assay units	Total no of bands	Number of polymorphic bands (%)	Number of bands per assay unit	Number of phenotypes per assay unit	Diversity index
RFLPs	114 (42 probes, 3 enzymes)	299	249 (83.2%)	2.62	2.37	0.322
RAPDs	22 (primers)	107	71 (66.3%)	4.86	3.41	0.521
SSRs	13 (primer pairs)	70	70 (100%)	5.38	5.38	0.566
AFLPs	6 (primer combinations)	297	139 (46.8%)	49.5	17.2	0.937

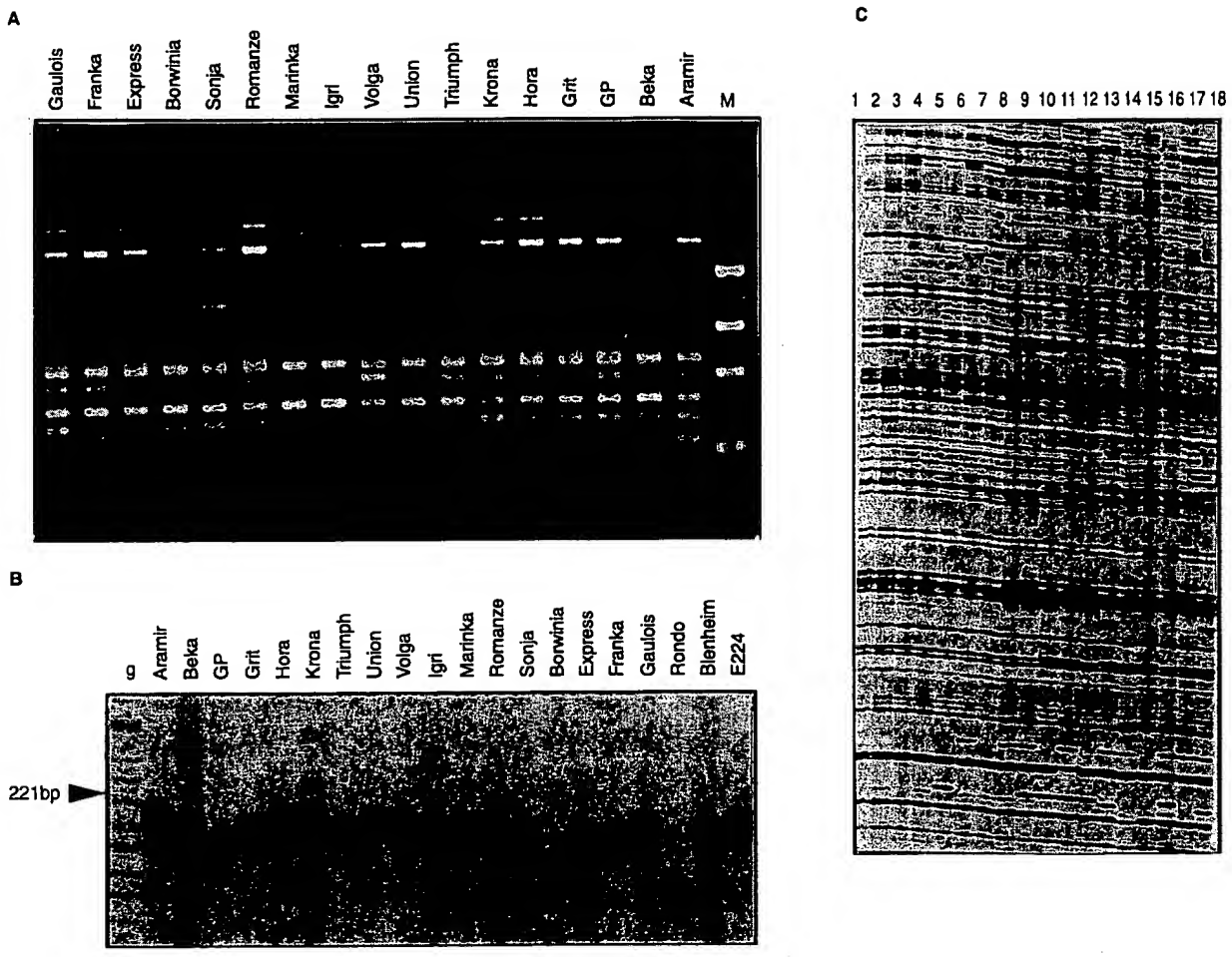

Fig. 1A–C An example of the different information content observed with RAPDS (A), SSRs (B) and AFLPs (C)

values for the comparisons of these 4 accessions with each of the molecular assays. With RFLPs, AFLPs and SSRs the genetic similarity values were higher than the mean values for all the spring types, and the highest similarity was between Triumph and Grit (RFLPs 0.93, AFLPs 0.97, SSRs 0.97). From the pedigree information in Table 1, Triumph and Grit share a number of parental lines including Union, Diamont and Hadm. With RAPDs, the genetic similarity values were less than the average, although the Triumph and Grit comparison was again the highest.

The genetic similarity values for the two-row winter varieties were intermediate between the spring and six-row winter types for RFLPs, AFLPs and SSRs. Sonja and Romanze were more similar than the other two-row winter types with values of 0.84 (SSRs), 0.93 (AFLPs), 0.89 (RFLPs) and 0.94 (RAPDs). This was not

Table 3 Maximum, minimum and mean genetic similarity estimates calculated from RFLP, RAPD, SSRs and AFLP data for winter and spring types

	RFLPs			RAPDs			AFLPs			SSRs			Parentage		
	Max	Min	Mean	Max	Min	Mean	Max	Min	Mean	Max	Min	Mean	Max	Min	Mean
Spring	93.0	76.0	84.3	95.0	84.0	87.9	97.0	88.0	92.4	93.0	66.0	82.9	0.330	0.020	0.133
Two-row winter	89.0	81.0	83.8	95.0	86.0	91.7	93.0	98.0	91.0	84.0	56.0	71.3	0.290	0.100	0.212
Six-row winter	85.0	60.0	70.0	95.0	88.0	89.7	91.0	81.0	87.7	90.0	45.0	65.7	0.350	0.000	0.111

Table 4 Genetic similarity values for the comparisons of 4 spring accessions with each of the molecular assays

	Grit	Krona	Triumph	Union	Maximum	Minimum	Mean
RAPDs:							
Grit	100.0						
Krona	88.0	100.0					
Triumph	90.0	85.0	100.0				
Union	88.0	88.0	84.0	100.0	95.0	84.0	87.9
RFLPs:							
Grit	100.0						
Krona	85.0	100.0					
Triumph	93.0	88.0	100.0				
Union	87.0	87.0	86.0	100.0	93.0	76.0	84.3
AFLPs:							
Grit	100.0						
Krona	91.0	100.0					
Triumph	97.0	92.0	100.0				
Union	93.0	94.0	94.0	100.0	97.0	88.0	92.4
SSRs:							
Grit	100.0						
Krona	84.0	100.0					
Triumph	97.0	87.0	100.0				
Union	69.0	70.0	94.0	100.0	97.0	66.0	82.9

unexpected as the co-efficient of parentage values were also the highest (0.290 for Sonja × Romanze compared to the mean for two-row winter type of 0.212). Both Sonja and Romanze are related through Malta, and Romanze has Sonja in its pedigree. The lowest genetic similarity values were observed for comparisons with Rondo.

Genetic relatedness

Associations among the 18 accessions were revealed by principal co-ordinate analysis (PCoA) (Fig. 2). The PCoA for the combined data (775 bands) clearly separated the winter from the spring accessions. Among the winter types, the two-rowed and six-rowed varieties formed two distinct groups, with the two-rowed types forming an intermediate group between the spring and six-rowed winter types. In the PCoAs generated by RFLP (299 bands) and AFLP (297 bands) data, a similar arrangement was observed. From the RAPD data, three distinct groups were again observed, although the spring types were more dispersed. Only a small group of spring types clustered together using SSR data, and two-row and six-row winter types were again more dispersed. On all of the PCoAs, Rondo appears in a remote position. In addition, 'Volga', a spring variety, was positioned between the rest of the spring and the two-rowed winter types.

Comparison between assays

To compare the results obtained with the four techniques, we tested correlations using Procrustes rotation (PR), linear regression of the pairwise GS values (LR) and Spearman rank correlation (SRC). The results for SRC (which compares how each system ranks pairwise similarities) are shown in Table 5. Comparisons using PR and LR showed the same general trends although the overall correlations were lower. Over 70% of the pairs of genotypes were ranked in the same order with RFLPs and AFLPs. This correlation is reduced to 10.9% when comparing RAPDs with AFLPs. SSRs were intermediate with over 50% of the genotypes ranking in the same order as that obtained with AFLPs and RFLPs.

Discussion

Given the proliferation of genetic markers, comparisons between techniques are inevitable. However, there is a need for such comparisons in order to decide on which technique is best suited to the issues being examined. In this study, three of the newer polymerase chain reaction (PCR)-based systems (RAPDs, SSRs and AFLPs) developed during the last 5 years have been compared with the well established RFLP system that was developed over 15 years ago. Each technique not only differs in principal, but also in the type and amount of polymorphism detected. The levels of polymorphism between the four techniques varied widely, ranging from a maximum of 100% (SSRs) to only 48.6% (AFLPs). Similar results were observed when Rus-Kortekaas et al. (1994) directly compared SSRs with RAPDs in tomato where the level of polymorphism was 40% with RAPDs compared to 100% with

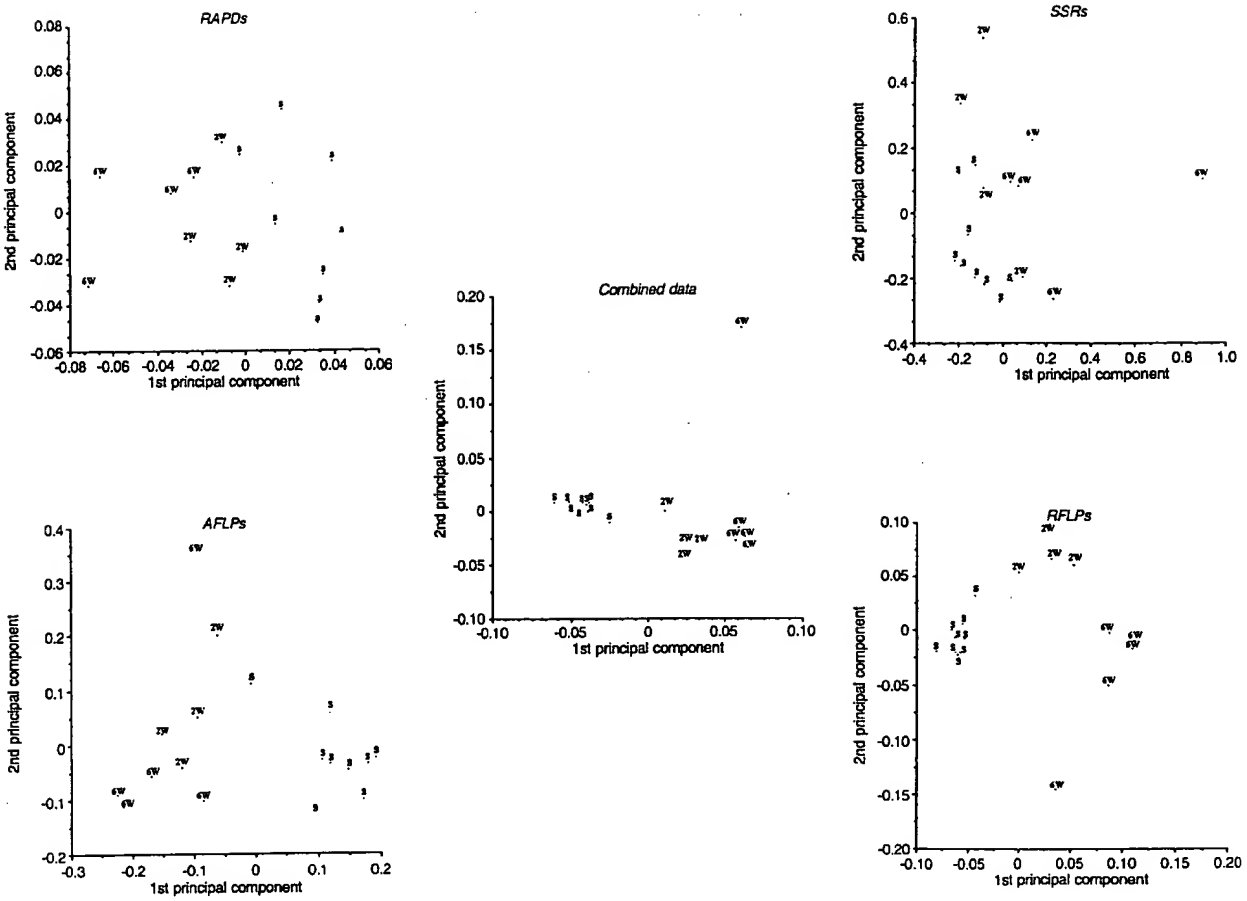

Fig. 2 Associations among the springs and winters cultivars revealed by principal co-ordinate analysis for each molecular assay

Table 5 Correlations obtained using RFLPs, RAPDs, AFLPs and SSRs based on Spearman's rank correlation and Procrustes rotation

SSR	1.000			
AFLP[a]	0.515	1.000		
RAPD[a]	0.235	0.109	1.000	
RFLP	0.505	0.708	0.201	1.000
	SSR	AFLP	RAPD	RFLP

[a] Rondo omitted from dataset for RAPD comparisons

SSRs. Indeed, whenever SSRs have been compared to other systems, they have always revealed the highest levels of polymorphism (Rus-Kortekaas et al. 1994; Salimath et al. 1995; Saghai Maroof et al. 1994; Powell et al. 1996; Maughan et al. 1995; Morgante et al. 1994; Wu and Tanksley 1993). The level of polymorphism detected using RFLPs in this study was higher (83.2%) than that observed in previous studies on barley using a similar selection of genotypes (46%) (Melchinger et al. 1994). This is probably due to pre-selection of polymorphic RFLP probes. The lowest level of polymorphism was associated with AFLPs. Becker et al. (1995) also observed that levels of polymorphism revealed by AFLPs were lower than by RFLPs. However, although AFLPs do not offer the highest level of polymorphism, they are the most efficient because they have the capacity to reveal many polymorphic bands in a single lane. The average number of bands per lane or per PCR for AFLPs was 49.5, compared to 1.0 band per lane or PCR for SSRs. Thus, when the overall diversity indices of the four techniques were compared, AFLP was the highest (0.937). Powell et al. (1996) introduced the concept of Marker Index as an overall measure of marker efficiency, and they demonstrated that, in *Glycine*, AFLPs had the highest Marker Index compared to other available marker systems. The high Marker Index or diversity index is a reflection of the efficiency of AFLPs to simultaneously analyse a large number of bands rather than the levels of polymorphism detected.

Barley germplasm can be divided into two genepools, winter and spring, based on morphology distinctions. Melchinger et al. (1994) using RFLPs observed a clear separation between the spring and winter

types. In this study, similar results were observed using RFLPs, AFLPs, RAPDs and SSRs. Furthermore, Melchinger et al. (1994) noted that sub-groups were also apparent for accessions with similar pedigrees, such as the compact grouping of two-row winter types intermediate between the spring and six-row winter types. With the exception of the SSR data, the two-row winter types form a sub-group between the six-row winter and the spring types for RFLP, AFLPs and RAPDs. With the SSR data there is a clear separation between the spring and winter types, but not within the winter types. This is not unexpected considering the low level of band sharing between accessions; even within groups the estimates of genetic similarity were much lower than any of the other assays.

Several previous studies have compared the use of RFLPs and RAPDs to examine genetic relatedness (Hallden et al. 1994; Thormann et al. 1994; Liu and Furnier 1993; dos Santos et al. 1994), and most of these show that RAPDs and RFLPs detect very similar relationships among the same group of accessions. Recently, other reports have compared RAPDs or RFLPs and SSRs on the same set of genotypes (Rus-Kortekaas et al. 1994; Wu and Tanksley 1993; Salimath et al. 1995; Maughan et al. 1995). Rus-Kortekaas et al. (1994) observed a lower percentage of band sharing in tomato accessions with SSRs compared to RAPDs and suggested that higher band sharing would make RAPDs more suitable for genetic relatedness studies. The results in this study would support the finding that SSRs may not be particularly well suited for pedigree relationship studies, although only a small number of SSRs were used.

Knowledge of genetic variation and the genetic relationship between genotypes is an important consideration for efficient rationalisation and utilisation of germplasm resources. Furthermore, it is important for the optimal design of plant breeding programmes, influencing the choice of genotypes to cross for the development of new populations. In barley, breeders have made crosses between highly selected genotypes with the result that the number of genotypes within the breeding genepool is very small. According to Graner et al. (1994) better knowledge and measures of genetic similarity of accessions could help to maintain genetic diversity. In the past, indirect estimates of similarity based on pedigree information have been widely used in many species including barley. Such estimates may not always reflect the true relationships between accessions (Graner et al. 1994). In this study we have used molecular markers to determine direct measures of genetic similarity between individuals. The estimates varied from 0.97 (AFLPs) to 0.45 (SSRs). Melchinger et al. (1994) reported GS values of 0.79 for unrelated barley pairs, based on RFLPs. The RFLP results reported in this paper were similar to these. Also, Tinker et al. (1993) observed GS values in a set of 27 North American barley cultivars using RAPDs which were similar to those found here (0.84–0.95). The values of GS based on SSRs in this present study are much lower than those based on RFLPs, AFLPs and RAPDs. Rus-Kortekaas et al. (1994) reported that the percentage of band sharing between tomato cultivars using SSRs was only 50.8% compared with 82.7% for RAPDs. Plaschke et al. (1995) observed even lower (0.31) estimates of genetic similarity when employing SSRs to examine wheat accessions and suggested that these low values are a reflection of the high information content provided by SSRs.

Although we have shown that molecular approaches can be used to group barley cultivars into morphologically distinct groups, and also further into sub-groups which have a similar genetic background, we have not addressed the issue of concordance of molecular-based estimates of GS and co-ancestry. Graner et al. (1994) compared RFLP-based estimates of GS with co-ancestry for a set of 48 cultivars. A very weak correlation was reported; $r_s = 0.21$ for winter and $r_s = 0.42$ for spring types. Similarly, using protein-based gliadin markers Cox et al. (1985) observed a correlation of $r_s = 0.27$. Both Graner et al. (1994) and Cox et al. (1985) agree that perhaps the reason for these poor correlations may be the high background similarity found for unrelated accessions using molecular markers. When related cultivars were used to investigate correlations between RAPD-based estimates of GS and co-ancestry a moderate correlation of $r_s = 0.61$ was observed between both measures (Tinker et al. 1993). Plaschke et al. (1995) observed similar results in wheat using SSR-based GS estimates and pedigree measures ($r_s = 0.55$). Although we have only a limited set of co-ancestry measures for the accessions studied here, several conclusions can be drawn from the correlations between molecular estimates of GS and the co-efficient of parentage. For example, the co-efficient of parentage for Rondo was 0 for all of the pairwise comparisons, and with all molecular measures Rondo had the lowest GS value. The low-to-moderate correlations between molecular measures of GS and pedigree estimates have led to the conclusion that pedigree information may not be as useful for certain applications for which they have been used in the past (Graner et al. 1994; Plaschke et al. 1995). In any case, molecular-based estimates of GS will provide more information than is available from pedigree information.

Having established that molecular-based estimates of GS will allow plant breeders to make informed decisions regarding the choice of genotypes to cross, we must ask the question as to which assay is most appropriate? Several studies have been described which address this question using isozymes, RFLPs and RAPDs (dos Santos et al. 1994; Thormann et al. 1994; Heun et al. 1994; Hallden et al. 1994). Heun et al. (1994) found that the correlation between RAPDs and isozymes among *Avena sterilis* accessions were moderately low

($r_s = 0.36$), although the overall representation of genetic relatedness was in considerable agreement. Beer et al. (1994) assessed genetic variation among *Avena sterilis* using morphological markers, isozymes and RFLPs and found a similarly low correlation ($r_s = 0.27$). A very different situation was observed among *Brassica* species. Thormann et al. (1994) reported correlations of $r_s = 0.969$ between RFLPs and RAPDs for a group of 18 accessions from different *Brassica* species. Dos Santos et al. (1994) also observed a significantly high correlation between RFLPs and RAPDs ($r_s = 0.745$) using genotypes within *Brassica oleracea*, although they did observe differences between the RFLP and RAPD dendrograms. When Spearman rank correlation was used, AFLPs and RFLPs ranked over 70% of the pairwise comparisons in the same order. This may well be because both techniques are based on restriction site changes, the major difference is that PCR is used in AFLPs rather than Southern analysis in RFLPs. In contrast SSRs and RAPDs have the lowest values when compared to the other assays. The low correlations observed with RAPDs could be a reflection of the choice of primers which we have previously used in the construction of a linkage map using a population derived from two related spring varieties. This may well have resulted in biased estimates of GS, which in turn has affected the ranking order of genotypes. For example, the lowest GS was between two spring types (Volga and Beka; GS = 0.84), whereas the lowest GS estimates for the other assays were between two winter types (even when Rondo was removed from all the data sets this still holds true).

The lack of correlation between SSRs and the other assays may not be fully unexpected, considering the high levels of polymorphism between pairwise comparisons. Powell et al. (1996) reported that SSRs were well-correlated with AFLPs and RFLPs at the interspecies level, however at the intraspecies level the correlation disappeared, emphasising the uniqueness of the SSR assay. Thus, while SSR analysis appeared to be the most polymorphic assay system, it did not seem to be particularly useful for assessing genetic relationships among cultivars. RFLPs were particularly valuable for assessing genetic relationships, but required several probe and enzyme combinations to discriminate between accessions. Both RFLPs and SSRs require an initial investment in terms of probe or sequence information, and according to Vos et al. (1995) the ideal fingerprinting assay should require no prior sequence knowledge. While only AFLPs and RAPDs meet these requirements, the lack of comparative information at each assayed locus (due to dominance) precludes an accurate assessment of true genetic relationships.

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haploid [Gk. *haploos,* single]: Having only one set of chromosomes (*n*), in contrast to diploid (2*n*).

hardwood: A name commonly applied to the wood of a dicot tree.

Hardy-Weinberg law: The mathematical expression of the relationship between the relative frequencies of two or more alleles in a population; it demonstrates that the frequencies of alleles and genotypes will remain constant in a random-mating population in the absence of inbreeding, selection, or other evolutionary forces.

haustorium, *pl.* **haustoria** [L. *haustus,* from *haurire,* to drink, draw]: A projection of fungal hypha that functions as a penetrating and absorbing organ.

heartwood: Nonliving and commonly dark-colored wood in which no water transport occurs; it is surrounded by sapwood.

heliotropism [Gk. *helios,* sun]: *See* solar tracking.

hemicellulose (hĕm'i-sĕl'u-lōs): A polysaccharide resembling cellulose but more soluble and less ordered; found particularly in cell walls.

herb [L. *herba,* grass]: A nonwoody seed plant with a relatively short-lived aerial portion.

herbaceous: An adjective referring to nonwoody plants.

herbarium: A collection of dried and pressed plant specimens.

herbivorous: Feeding upon plants.

heredity [L. *heredis,* heir]: The transmission of characteristics from parent to offspring through the gametes.

hermaphrodite [Gk. for Hermes and Aphrodite]: An organism possessing both male and female reproductive organs.

hetero- [Gk. *heteros,* different]: Prefix meaning "other" or "different."

heterocyst [Gk. *heteros,* different, + *cystis,* a bag]: A transparent, thick-walled, nitrogen-fixing cell that forms in the filaments of certain cyanobacteria.

heteroecious (hĕt'er-ē'shŭs) [Gk. *heteros,* different, + *oikos,* house]: As in some rust fungi, requiring two different host species to complete the life cycle.

heterogamy [Gk. *heteros,* other, + *gamos,* union or reproduction]: Reproduction involving two types of gametes.

heterokaryotic [Gk. *heteros,* other, + *karyon,* kernel]: In fungi, having two or more genetically distinct types of nuclei within the same mycelium.

heteromorphic [Gk. *heteros,* different, + *morphe,* form]: A term used to describe a life history in which the haploid and diploid generations are dissimilar in form.

heterosis [Gk. *heterosis,* alteration]: Hybrid vigor, the superiority of the hybrid over either parent in any measurable character.

heterosporous: Having two kinds of spores, designated as microspores and megaspores.

heterothallic [Gk. *heteros,* different, + *thallus,* sprout]: A term used to describe a species, the haploid individuals of which are self-sterile or self-incompatible; two compatible strains or individuals are required for sexual reproduction to take place.

heterotroph [Gk. *heteros,* other, + *trophos,* feeder]: An organism that cannot manufacture organic compounds and so must feed on organic materials that have originated in other plants and animals; *see also* autotroph.

heterozygous: Having two different alleles at the same locus on homologous chromosomes.

Hill reaction: The oxygen evolution and photoreduction of an artificial electron acceptor by a chloroplast preparation in the absence of carbon dioxide.

hilum [L. *hilum,* a trifle]: (1) Scar left on seed after separation of seed from funiculus; (2) the part of a starch grain around which the starch is laid down in more or less concentric layers.

histone: The group of five basic proteins associated with the chromosomes of all eukaryotic cells.

holdfast: (1) Basal part of a multicellular alga that attaches it to a solid object; may be unicellular or composed of a mass of tissue; (2) cuplike structures at the tips of some tendrils, by means of which they become attached.

homeo-, homo- [Gk. *homos,* same, similar]: Prefix meaning "similar" or "same."

homeostasis (hō'me-ō-stā'sis) [Gk. *homos,* similar, + *stasis,* standing]: The maintaining of a relatively stable internal physiological environment within an organism, or a steady-state equilibrium in a population or ecosystem. Homeostasis usually involves feedback mechanisms.

homokaryotic [Gk. *homos,* same, + *karyon,* kernel]: In fungi, having nuclei with the same genetic makeup within a mycelium.

homologous chromosomes: Chromosomes that associate in pairs in the first stage of meiosis; each member of the pair is derived from a different parent. Homologous chromosomes are also called homologues.

homology [Gk. *homologia,* agreement]: A condition indicative of the same phylogenetic, or evolutionary, origin, but not necessarily the same in present structure and or function.

homosporous: Having only one kind of spore.

homothallic [Gk. *homos,* same, + *thallus,* sprout]: A term used to describe a species in which the individuals are self-fertile.

homozygous: Having identical alleles at the same locus on homologous chromosomes.

hormogonium, *pl.* **hormogonia:** A portion of a filament of a cyanobacterium that becomes detached and grows into a new filament.

hormone [Gk. *hormaein,* to excite]: A chemical substance produced usually in minute amounts in one part of an organism, from which it is transported to another part of that organism on which it has a specific effect.

host: An organism on or in which a parasite lives.

humus: Decomposing organic matter in the soil.

hyaloplasm: *See* cytoplasmic ground substance.

hybrid: Offspring of two parents that differ in one or more heritable characteristics; offspring of two different varieties or of two different species.

hybridization: The formation of offspring between unlike parents.

hybrid vigor: *See* heterosis.

hydrocarbon [Gk. *hydro,* water, + L. *carbo,* charcoal]: An organic compound that consists only of hydrogen and carbon atoms.

hydrogen bond: A weak bond between a hydrogen atom attached to one oxygen or nitrogen atom and another oxygen or nitrogen atom.

hydrolysis [Gk. *hydro,* water, + *lysis,* loosening]: Splitting of one molecule into two by addition of the H^+ and OH^- ions of water.

hydrophyte [Gk. *hydro,* water, + *phyton,* a plant]: A plant that depends on an abundant supply of moisture or that grows wholly or partly submerged in water.

hydroxyl group: An OH^- group; a negatively charged ion formed by the dissassociation of a water molecule.

are no *flagella. They are *chemo-organotrophic, and can grow in the presence or absence of air. They are epiphytic (*see* epiphyte) or *parasitic in plants and arthropods, and can cause disease (e.g. stubborn disease of citrus trees).

Spirulina A genus of filamentous *cyanobacteria (section III) in which the filaments are characteristically spiral in shape. They are capable of gliding motility. They are found in freshwater and marine habitats, mostly in warmer regions; and are also found in hot springs. *Spirulina* is collected and used as food in the region of Lake Chad, Africa.

Splachnum (order *Funariales) A genus of mosses which are characteristically found growing on or associated with animal dung. The leaves are soft, lanceolate to obovate, with a thin nerve. The *capsule is held erect on a long *seta. *Splachnum* is a small genus found mainly in the northern hemisphere. *S. ampullaceum* and *S. sphaericum* occur in Britain. *S. ampullaceum* forms dense, light-green tufts on cattle dung. The capsules, borne on red setae, are often numerous and are characteristic in shape, resembling miniature Greek amphorae. They are found mainly in the upland regions of the west and north of Britain, particularly on wet *heaths and *moors, and in *bogs.

spleenwort *See Asplenium.*

spodic horizon A subsurface soil *horizon in which organic matter together with aluminium and often iron compounds have accumulated amorphously. It is a *diagnostic horizon in the *USDA Soil Taxonomy.

Spodosols An order of the *USDA Soil Taxonomy describing soils in which subsurface *horizons contain amorphous materials comprising organic matter and compounds of aluminium and often iron that have accumulated illuvially (*see* illuviation). Such soils form in acid material, mainly coarse in texture, in humid cool to temperate climates, often beneath coniferous forests.

Spondias (family *Anacardiaceae) A genus of trees, most of which have *pinnate leaves. The fruit is a slightly angled *drupe with a big round or angled stone, edible in several species (hog plum). There are 10 species occurring in Indo-Malaysia and tropical America.

spontaneous mutation In genetics, a naturally occurring *mutation, as opposed to one artificially induced by chemicals or irradiation. Usually such mutations are due to errors in the normal functioning of cellular *enzymes.

sporangiolum A *sporangium within which there is only a single *spore or a small number of spores.

sporangiophore A specialized *hypha which bears a *sporangium.

sporangiospore A *spore formed within a *sporangium.

sporangium A sac-like structure within which fungal *spores are formed; spores are liberated on rupture of the sporangium wall.

spore 1 (mycol.) A microscopic structure which functions in reproduction and dispersal. A spore does not contain an *embryo and is thus distinct from a *seed. Many different types of spore are produced by *fungi. 2 (bacteriol.) A differentiated cell which may function as a *propagule or as a resistant structure that allows the organism to survive adverse environmental conditions. *See also* heterospory; homospory; megaspore; microspore; and pollen.

spore mother cell A *diploid plant cell that gives rise to 4 *haploid *spores during *meiosis.

spore print The pattern of *spores obtained when the *cap of a fungal *fruit body is placed, *gills or pores down, on a sheet of paper and left for a period of time.

Sporobolomycetaceae (class *Blastomycetes) A family of *imperfect *yeasts characterized by the production of *ballistospores. These yeasts are *saprotrophic, and found on plant material.

sporocarp A structure the primary function of which is the production of *spores; a *fruit body. The term is applied particularly to the spore-producing structure of water ferns, which consists of a *sorus completely enclosed by the *indusium.

sporodochium A cushion-shaped mass of fungal *tissue densely covered with *conidiophores.

sporogenesis The production of *spores in plants. In *mosses and *liverworts the process occurs in a *sporogonium; in *fungi it occurs in a *sporophore; in other spore-forming plants it occurs in a *sporangium.

sporogonium In mosses (*Musci) and liverworts (*Hepaticae), the *sporophyte generation that develops after sexual reproduction and produces *spores.

sporogony A process of reproduction in which a *zygote undergoes multiple fission.

sporophore A structure upon which *spores are borne directly.

sporophyll A leaf in the *Pteridophyta that bears *sporangia. The term is most often applied to the small, scale-like leaves in the cones of *Lycopodium* and *Selaginella*, which bear *sporangia on their upper sides.

sporophyte The *spore-producing *diploid generation in the life cycle of plants. In higher plants, such as *angiosperms and *gymnosperms, the sporophyte is the dominant generation, forming the conspicuous plant. In lower plants, such as *mosses, *liverworts, *ferns, and *fungi, the *gametophyte is the dominant and conspicuous generation. *See also* alternation of generations.

sport A sudden deviation from type; a *mutation.

sporulation The process of *spore formation.

spraing A disease of potatoes, which may be caused by either of 2 different viruses, or by adverse growth conditions. *Tubers from infected plants show characteristic crescent-shaped brown marks in the flesh when cut. The viruses are transmitted by eelworms or by soil *fungi.

spray Wood trimmings, sold in faggots for kindling.

Concise Oxford Dictionary of Botany, ed. M. Allaby, Oxford Univ. Press pp 442 (1992)